REMARKS

This paper is submitted in response to the Office Action mailed September 22, 2003. Claims 1-6 are pending.

Priority

The Examiner states that the above-referenced application does not comply with one or more of the conditions for receiving the benefit of an earlier filing date under U.S. § 120 because the oath and declaration does not claim benefit to parent application, U.S. Appln. Serial No. 09/083,290 filed May 22, 1998.

In response, Applicants submit that the present application is a continuation of parent application, U.S. Appln. Serial No. 09/083,290. At the time of filing of the present application, the specification was amended to contain a specific reference to the earlier filed application. Furthermore, the filing receipt dated July 26, 2002 acknowledged the priority claim to U.S. Appln. Serial No. 09/083,290. In addition, a newly executed oath claiming benefit to the parent application is not required in a continuation application.

Applicants would also like to thank the Examiner for pointing out the inadvertent error made in the cross-reference to related application section. Applicants have amended the paragraph to specify priority only to parent application Serial No. 09/083,290, filed May 22, 1998, and delete the reference to the CPA application, filed July 5, 2001.

Oath and Declaration

The Examiner states that the Declaration is defective because it does not claim priority to parent application U.S. Appln. Serial No. 09/083,290. As indicated in the prior section, the present application is a continuation of parent application, U.S. Appln. Serial No. 09/083,290,

and, at the time of filing of the present application, the specification was amended to contain a specific reference to the earlier filed application.

Pursuant to 37 C.F.R. § 1.63(d), a newly executed oath or declaration is not required in a continuation application. At the time of filing of the present continuation application, Applicants submitted a copy of the executed Combined Oath and Declaration from the parent application U.S. Appln. Serial No. 09/083,290, to which the present application claims priority. Therefore, Applicants submit that the submitted oath or declaration is not defective.

Specification

The Examiner has requested correction of the first line of the specification. Applicants have amended the specification to claim priority to U.S. Appln. Serial No. 09/083,290.

The Examiner states that the Applicants are required to insert ATCC Accession Nos. at page 15. The Examiner also states that the Applicants are required to submit a declaration stating that they have maintained control and possession of the vectors since the filing date.

The vectors disclosed at page 15 were deposited with the ATCC in accordance with the Budapest Treaty, on May 19, 1998, prior to the filing date of the parent application U.S. Appln. Serial No. 09/083,290 of May 22, 1998. Enclosed is a copy of the ATCC International Form, dated June 24, 1998, acknowledging receipt of the deposited vectors. This information was supplied to the Patent Office in an Amendment mailed July 31, 2000 in the parent application. Applicants believe that since the deposit under the Budapest Treaty was made prior to the filing date of the parent application, there is no new matter introduced as a result of the insertion of the ATCC Accession Nos. and no declaration stating that the inventors have maintained control and possession of the vectors is necessary. However, if required, Applicants will supply such a declaration.

The 35 U.S.C. § 101 Rejection should be withdrawn

The Examiner has rejected pending Claims 1-6 under 35 U.S.C. §101 as unpatentable due to lack of utility. The Examiner alleges that the specification does not provide a function for the *Vezf1* or DB1 proteins. The Examiner also states that there is a 98% homology between *Vezf1* and DB1 and that the function of DB1 is unknown. Because the specification does not compare the homologies of *Vezf1* or DB1 with any protein with a known function, the Examiner alleges that the function of *Vezf1* or DB1 cannot be determined with any certainty. The Examiner states that while the DNA claimed in the invention may be used to make protein or to test for gene expression, the Examiner alleges that such a use is not of value if the function of the protein is unknown. The Examiner alleges that there is no apparent utility for the protein, if the *Vezf1* or DB1 genes do not have known functions.

Applicants respectfully disagree. An invention has a well-established utility if (1) a person of ordinary skill in the art would immediately appreciate that the invention is useful based on the characteristics of the invention (e.g. properties or application of a product or process), and (2) the utility is specific, substantial and credible. Furthermore, an applicant need only provide one credible assertion of specific and substantial utility for each claimed invention to satisfy the utility requirement.

First of all, the Examiner cannot use the absence of identified function attributed to DB1 gene products as a basis for the lack of utility in *Vezf1* genes and proteins. It is the function of *Vezf1* that is the focus of the present invention. *Vezf1* and DB1 are highly homologous, but independent genes. The relevant inquiry here is the utility of the *Vezf1* gene and gene products. A mere comparison with DB1 and its gene products does not preclude an analysis of the well-

established utility of the *Vezf1* gene and its gene products disclosed in the specification and discussed herein.

Secondly, Applicants disagree with the Examiner's contention that *Vezf1* should be compared with a sequence of a protein having a known function in order to determine the functionality of *Vezf1*. The expression data, as will be discussed below, disclosed in the instant specification is highly specific to vascular endothelial cells during development, implicating a functional role in vascular endothelial cell proliferation.

Thirdly, Applicants assert that the instant specification clearly discloses functions for the *Vezf1* gene products. They include the use of *Vezf1* as an endothelial cell marker (page 7, lines 11-13), its use in the development of animal models for angiogenesis (page 8, lines 1-6), its use as a diagnostic tool for vascular disorders (page 7, lines 14-21), and its use in the treatment of vascular disorders (page 8, lines 7-11). These uses described above are supported by the pattern of *Vezf1* expression in proliferating endothelial cells found in capillary networks and mature vessels (Figures 9-12; page 41, line 8 to page 43, line 11). Therefore, the Applicants assert that the present invention provides many sufficient examples showing the functionality of Vezf1.

The previously submitted Declaration of Dr. Heidi Stuhlmann also provides ample support for the functionality of *Vezf1*. Not only is *Vezf1* mRNA expression localized to endothelial cells, its expression is upregulated in injured endothelial cells. Injury-induced proliferation of endothelial cells in rat aortas stimulated *Vezf1* upregulation. *Vezf1* expression in the injured cells correlated with specific function that is related to angiogenesis. In addition, *Vezf1* expression was detected throughout the capillary network of tumors induced in nude mice by the introduction of human primary carcinoma cell lines. Specific *Vezf1* expression during

endothelial cell proliferation in normal and injured tissues demonstrates that possess functional characteristics in the regulation of angiogenesis or vasculogenesis.

In addition, analysis of the *Vezf1* amino acid sequence indicates that the protein contains six C2H2 zinc finger domains, characteristic of transcription factors (specification, page 39, line 21 to page 40, line 7). Taken together with the expression data, the structural analysis of the amino acid sequence implicates Vezf1 as a transcriptional regulator of vascular endothelial cells throughout development. In further support of its function as a transcriptional regulator, Aitsebaomo et al. have shown that *Vezf1* protein is localized to the nucleus and is capable of selectively transactivating the endothelial cell-specific human endothelin-1 promoter *in vitro* (Aitsebaomo et al. 2001, "*Vezf1*/DB1 Is an Endothelial Cell-specific Transcription Factor That Regulates Expression of the Endothelin-1 Promoter," Journal of Biological Chemistry, 276:39197-39205, p. 39199; attached as Exhibit 1).

Furthermore, recent data in knockout *Vezf1* mice show that *Vezf1* possesses tight control over vascular development, retention of vascular integrity, and lymphatic development (Kuhnert et al. "*Vezf1* Function is Required for Murine Vascular Development and Haploinsufficiency REsults in Lymphatic Hypervascularization," 2004, submitted manuscript; attached as Exhibit 2). Inactivation of *Vezf1* in homozygous knockouts lead to embryonic lethality, with the embryos exhibiting intraembryonic hemorrhaging (Kuhnert et al.; Figures 2 and 4). Heterozygous mutant embryos showed edema and hemorrhaging in the back and neck areas, hypervascularization in the jugular region, and loss of vascular integrity (Kuhnert et al.; Figure 5). Heterozygous mutant embryos also exhibited hypervascularization of the lymphatics in the jugular region (Kuhnert et al.; Figure 7). These results show clear functional roles for *Vezf1* gene products that were contemplated by the instant specification.

Given the specificity of the expression data disclosed in the specification and the confirmation of many functional characteristics through cellular and animal data, Applicants assert that claimed *Vezf1* gene has patentable utility.

If a patent discloses only nucleic acid molecular structure for a newly discovered gene, and no utility for the claimed isolated gene, the claimed invention is not patentable. But when the inventor also discloses how to use the purified gene isolated from its natural state, the application satisfies the "utility" requirement (Utility Examination Guidelines, Federal Register Vol. 66, No. 4, January 5, 2001, p 1093). Applicants submit that the specification has provided more than adequate support for ways of using the purified gene from its natural state, including, *inter alia*, as an endothelial cell marker, as a diagnostic tool for vascular disorders, incorporation into cloning or expression vectors, production of monoclonal or polyclonal antibodies against the *Vezf1* protein. Therefore, Applicants submit that claims 1-6 are patentable under 35 U.S.C. § 101.

Applicants also contend that a person of ordinary skill in the art would immediately appreciate that the present invention is useful based on the spacial and temporal expression pattern of *Vezf1* and that the present invention satisfies the requirement for credible utility under 35 U.S.C. §101 and respectfully request withdrawal of the rejection.

Finality of the Office Action

The outstanding Office Action dated September 22, 2003 is the first Office Action issued in the present application. Although the Office Action summary page and the PAIR status page indicates that this Office Action is non-final, pages 4 and 5 of the Office Action indicate that the Office Action is made final. Examiner Wilson was contacted by telephone on March 9, 2004 to

clarify the discrepancy. Examiner Wilson confirmed that the Office Action was non-final and agreed to issue an Interview Summary to this end.

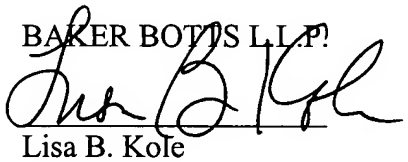
CONCLUSION

In view of the foregoing remarks, Applicants respectfully request withdrawal of the outstanding rejections and allowance of the pending claims.

Applicants request a three month extension of time and enclose herewith the requisite fee as set forth in 37 C.F.R. § 1.17(a)(3). Applicants do not believe that any additional fee is required in connection with the submission of this document. However, should any fee be required, or if any overpayment has been made, the Commissioner is hereby authorized to charge any fees, or credit or any overpayments made, to Deposit Account 02-4377. A duplicate copy of this sheet is enclosed.

Respectfully submitted,

BAKER BOTTS L.L.P.



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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 42, Issue of October 19, pp. 39197–39205, 2001
Printed in U.S.A.

Vezf1/DB1 Is an Endothelial Cell-specific Transcription Factor That Regulates Expression of the Endothelin-1 Promoter*

Received for publication, June 5, 2001, and in revised form, August 9, 2001
Published, JBC Papers in Press, August 14, 2001, DOI 10.1074/jbc.M105166200

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Coordinated gene regulation within the vascular endothelium is required for normal cardiovascular patterning during development and for vascular homeostasis during adulthood, yet little is known about the mechanisms that regulate endothelial transcriptional events. Vascular endothelial zinc finger 1 (Vezf1)/DB1 is a recently identified zinc finger-containing protein that is expressed specifically within endothelial cells during development. In this report, we demonstrate that Vezf1/DB1 is a nuclear localizing protein that potently and specifically activates transcription mediated by the human endothelin-1 promoter, in a Tax-independent manner, in transient transfection assays. Using a combination of deletion mutagenesis and electrophoretic mobility shift assays, a novel Vezf1/DB1-responsive element was localized to a 6-base pair (bp) motif, ACCCCC, located 47 bp upstream of the endothelin-1 transcription start site. Recombinant Vezf1/DB1 also bound to this sequence, and a 2-bp mutation in this element abolished Vezf1/DB1 responsiveness by the endothelin-1 promoter. Vezf1/DB1 could be identified with a specific antibody in nuclear complexes from endothelial cells that bound to this element. Regulation of endothelin-1 promoter activity by Vezf1/DB1 provides a mechanism for endothelin-1 expression in the vascular endothelium during development and to maintain vascular tone; Vezf1/DB1 itself is a candidate transcription factor for modifying endothelial cell phenotypes in order to appropriately assemble and maintain the cardiovascular system.

The endothelium maintains vascular integrity during adulthood by regulating vascular tone, lymphocyte trafficking, vessel growth, and hemostasis (1). In particular, the vascular endothelium modulates vascular tone and blood pressure through the coordinated production of potent vasoactive molecules. In addition, the endothelium directs formation of the vascular system during development through its role in the processes of *vasculogenesis* (development of blood vessels from angioblasts *in situ*) and *angiogenesis* (sprouting of new vessels from existing vessels) (2). Whereas endothelial mechanisms during development and in adulthood may seem discrete, there is now strong evidence that they overlap considerably, and that molecules required for normal vascular development may contribute to vascular homeostasis in adulthood, and vice versa.

Endothelin-1 (ET-1),[1] a 21-amino acid peptide originally isolated from porcine endothelial cells (3), provides one example of an endothelial cell protein that is critical both for cardiovascular development in the embryo and for vascular homeostasis in the adult. ET-1, which is expressed primarily in endothelial cells and also epithelial cells of the branchial arches during development (4), is required for the development of neural crest-derived tissues arising from the branchial arches, such as the great vessels, the ventricular septum, and craniofacial structures (5). These effects seem to be mediated via interactions with the endothelin-A receptor, which is expressed in neural crest cells. Loss of ET-1 signaling leads to apoptosis of post-migratory neural crest-derived mesenchymal cells, with resultant cardiovascular defects such as ventricular septal defects, truncus arteriosus, and interruption of the aortic arch.

In addition to its function as a critical developmental signal for the cardiovascular system, ET-1 has potent and complex effects on the adult vasculature. ET-1 has direct vasoconstrictor effects on vascular smooth muscle cells, and sensitizes smooth muscle cells to the effects of angiotensin II and norepinephrine (6). ET-1 also stimulates the release of aldosterone and nitric oxide, the latter effect indicating that the vasoconstrictor effects of ET-1 may be most pronounced when endothelial function is compromised. The effects of ET-1 are linked pathophysiologically to the development of hypertension, cardiac hypertrophy (7), and ischemic heart disease (8).

Given the bifunctional role of ET-1 in development and in vascular homeostasis, it is not surprising that its expression and activity are tightly regulated at the transcriptional level. The human *ET-1* gene has a TATA box-containing promoter, and several *cis*-acting elements have been implicated in transcriptional regulation of ET-1 mRNA. An upstream activator protein-1 (AP-1) site located at position −117, which is bound by c-*fos* and c-*jun*, is required for inducible high-level *ET-1* promoter activity (9). In addition, a GATA motif at position −135, which is bound by the zinc finger transcription factor GATA-2 in endothelial cells (10), is crucial to the basal and

* This work was supported by NHLBI National Institutes of Health Grants HL03658 and HL61656 (to C. P.) and by a National Research Service Award (to J. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: *ET-1*, endothelin-1; Vezf1, vascular endothelial zinc finger 1; AP-1, activator protein 1; HIF-1, hypoxia inducible factor 1; *hET-1*, human endothelin-1; MEC, myocardial endothelial cells; GST, glutathione *S*-transferase; GFP, green fluorescent protein; CHIP, carboxyl terminus of Hsc70-interacting protein; CMV, cytomegalovirus; bp, base pair(s); EMSA, electrophoretic mobility shift assay.

regulated expression of the *ET-1* gene. Furthermore, the cooperativity between the AP-1 complex and GATA-2 leads to a synergistic increase in *trans*-activation of *ET-1* (11). Last, hypoxia-mediated expression of ET-1 is mediated via *trans*-activation of the *ET-1* promoter by hypoxia-inducible factor-1 (HIF-1) (12). However, the expression of *fos*/*jun* and GATA-2 is not restricted to endothelial cells, and the up-regulation of the *ET-1* gene via the GATA motif is not limited to GATA-2, as other members of the GATA family, such as GATA-1 and GATA-3, exert a similar effect (11). Because GATA-2, HIF-1, and the Fos and Jun family members are expressed more promiscuously than is ET-1, the action of these factors alone cannot be responsible for the cell type-restricted activation of the *ET-1* gene. Therefore, it is possible that the binding of the *trans*-acting factors to these sites may recruit or otherwise cooperate with additional proteins which are important for cell restricted expression of the *ET-1* gene. In any event, a 6-kilobase fragment of the mouse *ET-1* promoter confers vascular-specific expression in transgenic mice (13).

Vezf1/DB1 is a recently identified endothelial cell-specific protein. A retroviral trap screen identified a 56-kDa protein expressed specifically in the vascular endothelium, vascular endothelial zinc finger 1 (Vezf1) (14). Vezf1, which is the mouse homologue of a previously identified but incompletely characterized human protein called DB1 (15), is a putative transcription factor that contains 6 Cys_2/His_2-type zinc finger motifs, as well as a glutamine-stretch and a proline-rich region characteristic of transcriptional activation or repression domains. Vezf1/DB1 is first expressed in the anterior-most mesoderm at day 7.25 post-conception. Expression remains restricted to the vascular endothelium through at least day 13.5 and is detectable in endothelial cells undergoing both angiogenesis and vasculogenesis (14). Vezf1/DB1 is therefore an attractive candidate as a potential transcription factor for mediating endothelial cell-specific gene expression, and is expressed in the correct spatial and temporal sequence during embryogenesis to regulate genes critical for endothelial cell differentiation, cardiovascular development, and/or angiogenesis. In an effort to identify transcriptional targets for Vezf1/DB1 in vascular endothelial cells, we have found that Vezf1/DB1 potently *trans*-activates the human endothelin-1 (*hET-1*) promoter, and we have characterized a novel Vezf1/DB1-responsive element in the *ET-1* 5′-flanking sequence.

MATERIALS AND METHODS

Plasmids—Plasmids pGL2-Basic and pGL2-Control contain the firefly luciferase gene (Promega). pGL2-Basic lacks a promoter, whereas pGL2-Promoter expresses luciferase under control of the SV40 promoter. The plasmid pCMV-βGal (Stratagene) contains the β-galactosidase gene driven by the CMV promoter.

Reporter constructs containing fragments of the *hET-1* 5′-flanking region were inserted into pGL2-Basic and named according to the length of the fragment (from the transcriptional start site) in the 5′ and 3′ directions. For example, plasmid pGL2–204/+170 contains a *hET-1* promoter fragment extending from −204 bp 5′ of the transcriptional start site to position +170 inserted into pGL2-Basic. Plasmids pGL2–204/+170, pGL2–143/+170, pGL2–129/+170, pGL2–95/+170, and pGL2–42/+170 were created by cloning the *Eco*RI/*Bgl*II fragment of plasmids p-204CAT, p-143CAT, p-129CAT, p-95CAT, and p-42CAT, respectively (16), in the appropriate orientation upstream of the luciferase gene of plasmid pGL2-Basic. These constructs share a common 3′ *Bgl*II site but differ at the 5′ end located at base pairs −204, −143, −129, −95, and −42, respectively.

pGL2-KDR/flk-1 contains a fragment of the KDR/flk-1 promoter from base pairs −570 to +268, and has been previously described (17). pME18S-DB1 was created by cloning the *Pvu*II/*Not*I-digested fragment of IMAGE clone number 2114422 (Research Genetics), which contains the full-length human Vezf1/DB1 cDNA, into mammalian expression plasmid pME18S. $p40^{Tax}$, a Tax expression plasmid under control of SV40 promoter, was a generous gift from Kuan-Teh Jeang (NIAID, National Institutes of Health, Bethesda, MD) and has been described elsewhere (18).

Mutagenesis—Site-directed mutagenesis of the *hET-1* promoter was performed by polymerase chain reaction as described elsewhere (17) to create the plasmid pGL2–204/+170 mut-3. A DNA fragment containing *hET-1* promoter (pGL2–204/+170) was used as a template. The sequence TTACCCCCACTC was mutated to TTACATCCACTC using the mismatched primers 5′-GTCAGAGCTGTTTACATCCACTCTATAGGGGTTC-3′ and 5′-GAACCCCTATAGAGTGGATGTAAACAGCTCTGAC-3′. The sequence of the mutated polymerase chain reaction fragment was confirmed by the dideoxy chain termination method.

Cell Culture—The mouse myocardial endothelial cells (MEC), which were a kind gift from Robert Auerbach (University of Wisconsin), have been described elsewhere (19). NIH/3T3 and C2C12 cells were obtained from the Tissue Culture Facility at Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC. These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (or calf serum for NIH/3T3 cells), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Transfection and Luciferase Assays—MEC, C2C12, and NIH/3T3 cells were used for transient transfection experiments. Cells were grown overnight to 70–80% confluence in 6-well plates each containing 2 ml of Dulbecco's modified Eagle's medium at 37 °C. Cells were transfected with 1.0 μg of pGL2–204/+170, pGL2–204/+170 mut-3, pGL2–143/+170, pGL2–129/+170, pGL2–95/+170, pGL2–42/+170, pGL2-Promoter, pGL2-Basic, or pGL2-KDR/flk1 and the indicated concentration of pME18S-DB1 and/or $p40^{Tax}$ plasmid using the LipofectAMINE method (Life Technologies). pME18S was used to normalize the total concentration of plasmid. To correct for variability in transfection efficiency, 0.25 μg of pCMV-βGal was co-transfected in all experiments. 48 h after transfection, cells were lysed with 150 μl of 1 × reporter lysis buffer (Promega) according to the manufacturer's recommendation, and 50 μl of cell extracts were used for the luciferase assay. 30 μl of cell extract were used for the β-galactosidase assay as previously described (17). The ratio of luciferase activity to β-galactosidase activity in each sample served as a measure of the normalized luciferase activity. The normalized activity was expressed as fold-induction. Each construct was transfected at least 4 times, and data for each construct are presented as the mean ± S.E.

Recombinant Protein Production—A Vezf1/DB1 glutathione *S*-transferase (GST) fusion protein was created by inserting the Vezf1/DB1 cDNA in-frame in vector pGEX3 (Amersham Pharmacia Biotech) to create the plasmid pGEX-Vezf1/DB1. Recombinant protein was expressed in BL21-Gold(DE3) bacterial cells (Stratagene). Cells were grown at 37 °C to A_{600} 0.4, then isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM. Cells were grown at 30 °C for an additional 4 h. Bacteria were lysed by sonification, and GST-Vezf1/DB1 or GST was purified by affinity chromatography on glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The protein concentration of recombinant protein was determined by a modified Lowry procedure (DC protein assay, Bio-Rad) and confirmed by 10% sodium dodecyl sulfate-polyacrylamide gel fractionation followed by Coomassie Blue staining.

Intracellular Localization—Full-length Vezf1/DB1 cDNA was inserted into the green fluorescent protein (GFP) fusion vector pEGFP-C3 (CLONTECH). GFP-CHIP has been previously described (20), and expresses a GFP fusion with carboxyl terminus of Hsc70-interacting protein (CHIP). Plasmid pEGFP-C3, expressing GFP alone, served as a control for nonlocalized expression. Plasmids (1 μg) were transfected into MEC by the LipofectAMINE method. After 24 h, cells were examined for GFP expression by direct epifluorescence using a Nikon Diaphot 300 microscope.

Preparation of Nuclear Extracts—All buffers used in the nuclear extract preparation contained the complete EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals) and 5 mM sodium fluoride, 1 mM sodium vanadate, 1 mM sodium molybdate, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. MEC were grown in Dulbecco's modified Eagle's medium to confluence in 150-mm plates. Cells were washed with cold phosphate-buffered saline, then 1 ml of cold phosphate-buffered saline was added to each plate, scraped with a rubber policeman, and the cell content collected in 1.5-ml Eppendorf tubes on ice and centrifuged at 5000 rpm for 30 s at 4 °C. The supernatant was aspirated and cells were resuspended in 800 μl of hypotonic buffer (10 mM HEPES, pH 7.9, at 4 °C, 1.5 mM $MgCl_2$, 10 mM KCl). Cells were centrifuged at 5000 rpm for 30 s at 4 °C; the supernatant was discarded and cells were resuspended in 600 μl of hypotonic buffer and

incubated on ice for 10 min. Cells were vortexed for 10 s, and centrifuged at 5000 rpm for 30 s. The supernatant was discarded and the pellet was resuspended in 80 μl of extraction buffer (20 mM HEPES, pH 7.9, at 4 °C, 25% glycerol, 0.5 M KCl, 0.75 mM $MgCl_2$, 0.2 mM EDTA). The mixture was incubated for 45 min at 4 °C for nuclear extraction followed by ultracentrifugation and collection of the supernatant. The supernatant was dialyzed for 30 min in dialysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA), aliquoted, and snap frozen at −80 °C.

Antisera—Polyclonal antisera were generated in rabbits against a peptide (MEANWTAFLFQAHEC) corresponding to a conserved region of Vezf1/DB1 coupled to keyhole limpet hemagglutinin by standard procedures as previously described (20). The IgG fraction was purified from whole immune sera using Affi-Gel 10 (Bio-Rad) packed in a purification column as described in Affinity Purification of Antibodies from Crude Serum (www.protocol-online.net/immuno/antibody/antibody_purification.htm).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed as previously described (21). The probe consisted of annealed synthetic 54-bp complementary oligonucleotides corresponding to −95 to −42 of the *hET-1* 5′-flanking sequence (13) or a portion of the aforementioned *hET-1* 5′ flanking sequence corresponding to −95 to −67 (5′ probe), −76 to −57 (middle probe), or −66 to −42 (3′ probe) as shown in Fig. 5*A*. The synthetic oligonucleotides used for competition were the same oligonucleotides or mutated versions of the 3′ probe as shown in Fig. 6*A*. A typical binding reaction contained 75,000 cpm of DNA probe, 1.0 μg of poly(dI-dC)·poly(dI-dC), 5 μl of dialysis buffer (20 mM HEPES, pH 7.9, 10% glycerol, 100 mM KCl, and 0.2 mM EDTA), 0.6 μl of bovine serum albumin (10 mg/ml), 10 μg of nuclear extract, or 400 ng of recombinant Vezf1/DB1 in a final volume of 25 μl. The reaction mixture was incubated at room temperature for 25 min and fractionated on a 4% polyacrylamide gel in 1 × Tris glycine buffer. To determine the specificity of the DNA-protein complexes, we performed competition assays using a 50-fold molar excess of the unlabeled wild-type oligonucleotides (specific competitors), mutated oligonucleotides, or an unrelated CT/GC-rich double-stranded oligonucleotide of comparable length (5′-CCCACCCTGGTGCGAGTC-3′) as a nonspecific competitor. To characterize specific DNA-binding proteins, we incubated nuclear extracts with affinity purified rabbit polyclonal anti-Vezf1/DB1 antibody or a similarly prepared antibody to the unrelated sequence QEKLISEENL, or anti-GST antibody (Sigma) for 3 h at 4 °C before adding probe.

RESULTS

Vezf1/DB1 Is an Endothelial Cell-specific Zinc Finger Protein That Localizes to the Nucleus—Analysis of primary amino acid sequence of Vezf1/DB1 demonstrates that it contains 6 Cys_2/His_2-type zinc finger motifs and a proline-rich region characteristic of transcriptional activation or repression domains (14, 15), suggesting that Vezf1/DB1 is likely to function as a transcription factor to regulate endothelial cell-specific genes. As the first step toward determining the function of this protein, we characterized the intracellular localization of Vezf1/DB1. We expressed a fusion of full-length Vezf1/DB1 with GFP by transient transfection of MEC to localize expression of Vezf1/DB1 within the cell. As controls, we also expressed GFP alone and GFP fused to CHIP, a protein that we have previously shown to localize exclusively to the cytoplasm in this assay (20). Consistent with our previous results, GFP-CHIP localized to the cytoplasm (Fig. 1, *middle panels*), and GFP alone exhibited diffuse expression throughout the cell (*lower panels*). However, GFP-Vezf1/DB1 localized exclusively to the nucleus (*upper panels*). These results are consistent with previous assumptions that Vezf1/DB1 is a nuclear-localizing transcription factor, and led us to search for potential endothelial cell-specific transcriptional targets for this protein.

Vezf1/DB1 Specifically Trans-activates the hET-1 Promoter in Transient Transfection Assays—In order to identify potential transcriptional targets of Vezf1/DB1, we tested known endothelial cell-specific promoters to determine if they are *trans*-activated by Vezf1/DB1 in transient transfection assays. In our initial experiments, the reporter plasmids pGL2-Basic, pGL2-Promoter, pGL2-KDR/flk-1, or pGL2–204/+170 (expressing a fragment of the *hET-1* promoter) were co-transfected with Vezf1/DB1 into MEC. Co-transfection of Vezf1/DB1 had no effect on activity of the SV40 promoter (present in pGL2-Promoter) or on the endothelial cell-restricted promoter KDR/flk-1 (17) (Fig. 2*A*). In contrast, Vezf1/DB1 significantly *trans*-activated the *hET-1* promoter, another endothelial cell-restricted promoter (16), by ~40–50-fold. The *trans*-activation of *hET-1* promoter was dose-dependent and occurred over a broad range of plasmid concentrations (Fig. 2*B*). These results demonstrate that Vezf1/DB1 functions as a transcriptional activator and provide a rationale for further characterization of the mechanisms of *trans*-activation of the *hET-1* promoter by Vezf1/DB1.

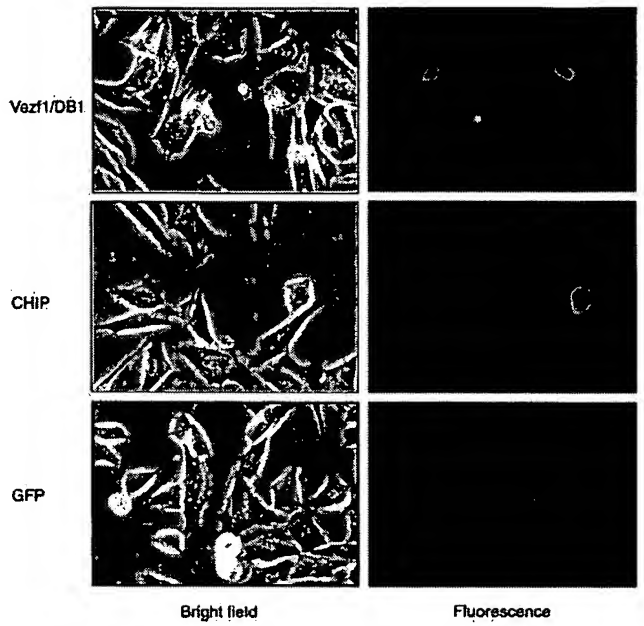


FIG. 1. **Intracellular localization of Vezf-1/DB1.** MEC were transiently transfected with GFP fusion plasmids, and localization was assessed by direct epifluorescence 48 h after transfection. *Upper right*, expression of GFP-Vezf1/DB1 fusion by epifluorescence with nuclear localization; *upper left*, the same field analyzed by light microscopy; *middle right*, expression of GFP-CHIP demonstrating cytoplasmic localization; *lower right*, GFP alone demonstrating both nuclear and cytoplasmic localization. Original magnification × 100.

Tax Does Not Potentiate Vezf1/DB1 Responsiveness by the ET-1 Promoter—The human homologue DB1 was originally cloned via potential interactions with a CT/GC-rich region in the interleukin-3 promoter (15). This same CT/GC-rich region in the interleukin-3 promoter has been defined as a responsive element for Tax-mediated *trans*-activation (22). Although DB1 did not have any direct transcriptional effects on the interleukin-3 promoter, DB1 augmented Tax-dependent transcriptional activity of interleukin-3 promoter by ~3-fold in a phorbol ester-dependent manner (15). In order to determine whether the mechanisms of *trans*-activation of the *hET-1* promoter by Vezf1/DB1 were different or similar to its effects on the interleukin-3 promoter, we asked whether similar functional interactions between Vezf1/DB1 and Tax occurred to modulate transcription of the hET-1 promoter. We co-transfected MEC with $p40^{Tax}$ and Vezf1/DB1. As expected, Vezf1/DB1 *trans*-activates the *hET-1* promoter; however, in contrast to its effects on the interleukin-3 promoter, co-transfection of Tax caused a dose-dependent repression of the transcriptional activity of Vezf1/DB1 (Fig. 3). These experiments emphasize that the effects of Vezf1/DB1 on *hET-1* promoter activity are distinct in several respects from previous experiments using the interleukin-3 promoter, in which Vezf1/DB1 seemed to function primarily as a transcriptional co-activator for Tax rather than as a direct *trans*-activator.

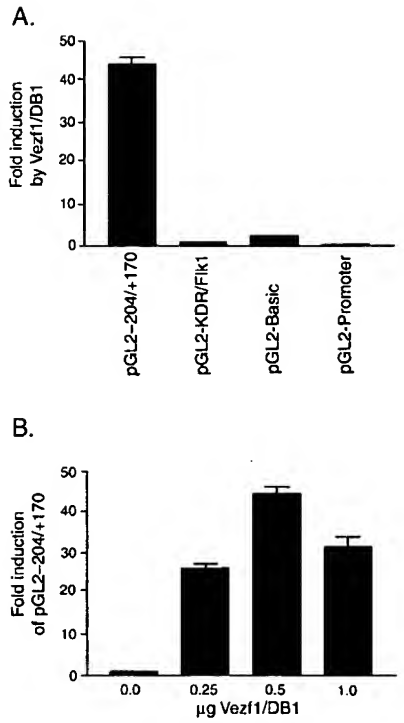


FIG. 2. **Trans-activation of *hET-1* by Vezf1/DB1.** *A*, 0.5 μg of Vezf1/DB1 was transfected with 1.0 μg of pGL2–204/+170 (containing a fragment of the human endothelin-1 promoter), pGL2-KDR/flk-1, pGL2-Basic, or pGL2-Promoter into MEC. The vector pCMV-βGal was used to correct for differences in transfection efficiency. Luciferase activity is expressed as fold induction relative to transfection in the absence of Vezf1/DB1. The results are expressed as the mean ± S.E. *B*, luciferase reporter construct pGL2–204/+170 was transiently transfected with the indicated concentrations of Vezf1/DB1 expression vector into MEC, and fold-induction is expressed relative to transfection in the absence of Vezf1/DB1.

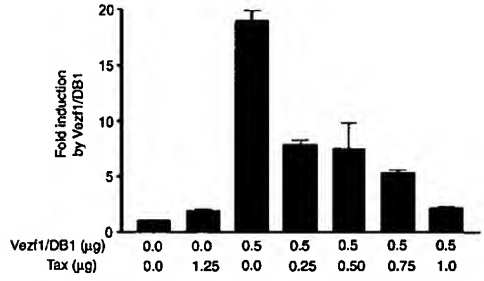


FIG. 3. **Effects of Tax on Vezf1/DB1-mediated trans-activation of the human endothelin-1 promoter.** Luciferase reporter construct pGL2–204/+170 was transiently transfected with the indicated concentrations of Vezf1/DB1 or Tax expression vector into MEC. The vector pCMV-βGal was used to correct for differences in transfection efficiency. Luciferase activity is expressed as fold induction relative to transfection in the absence of Vezf1/DB1. The results are expressed as the mean ± S.E.

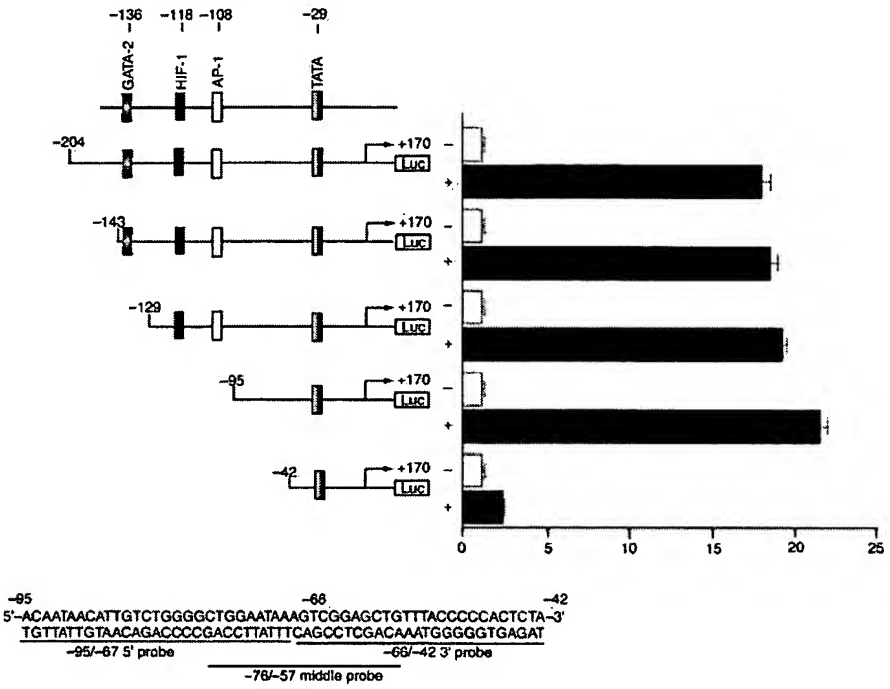
Identification of the Vezf1/DB1 Response Element by Deletion Analysis of the ET-1 Promoter—The transcription assays discussed above demonstrate that the sequence spanning −204/+170 of the *hET-1* 5′-flanking sequence is potently *trans*-activated by Vezf1/DB1. This *ET-1* promoter construct contains GATA-2, AP-1, TATA-binding protein, and HIF-1-binding sites (Fig. 4). In order to determine the Vezf1/DB1 response element within the *ET-1* 5′-flanking sequence, we tested serially deleted truncations from base pairs −204 to −42 (relative to the transcriptional start site) of the *ET-1* promoter to determine the specific sequences that are Vezf1/DB1-responsive. Deletion constructs from base pairs −204 to −95 were equally *trans*-activated by Vezf1/DB1, whereas further deletion to bp −42 totally abolished Vezf1/DB1-responsiveness (Fig. 4). These experiments thus localize the Vezf1/DB1-responsive element to a 54-bp sequence between −95 and −42 within the *hET-1* 5′-flanking sequence.

Characterization of a Nuclear Protein-binding Activity within the Vezf1/DB1 Responsive Element—Having identified the putative Vezf1/DB1-responsive element within the *hET-1* promoter via transient transfection assays, we sought to refine the location of this response element, and to determine whether nuclear proteins in general, and Vezf1/DB1 specifically, bound to these sequences. As a first step, we determined whether MEC contain nuclear binding activities that will associate with the fragment from −95 to −42 of the *hET-1* 5′-flanking sequence by EMSA. MEC were a convenient cell type to utilize for these experiments, as we found them to express Vezf1/DB1 constitutively (data not shown). A ^{32}P-labeled synthetic probe containing base pairs −95 to −42 or portions of it consisting of a series of overlapping oligonucleotides from base pairs −95 to −67 (5′ probe), −76 to −57 (*middle probe*), or −66 to −42 (3′ probe) were used to probe MEC nuclear extract (Fig. 5*A*). Incubation of radiolabeled double-stranded oligonucleotide consisting of base pairs −95 to −42 with nuclear extract from MEC identified several DNA-protein binding complexes (Fig. 5*B*), 3 of which could be specifically competed away with a 50-fold molar excess of the unlabeled full-length (*FL*) or 3′ probe competitors but not by an excess of the 5′ or middle nonspecific competitors. This experiment indicated that specific DNA/protein interactions could be detected within the Vezf1/DB1 response element, and that these interactions most likely occurred within the 3′ region of this sequence.

To further characterize nuclear protein binding within the Vezf1/DB1 response element, the 5′, middle, or 3′ probes were separately radiolabeled and used in a similar EMSA experiment (Fig. 5*C*). Only nonspecific binding activities were observed with the 5′ and the middle probes. However, the 3′ probe showed both specific and nonspecific binding activities that essentially recapitulate the binding activities seen in Fig. 5*B*. (We have labeled these binding activities *A*, *B*, and *V*. The fastest migrating binding activity, *V*, is variably present in our reactions and likely represents a labile activity or a degradation product derived from a component of one of the slowly migrating activities.) In addition to confirming the results of Fig. 5*B*, this experiment further localizes nuclear protein binding to the distal portion of the 3′ probe, a 16-bp region between bps −57 to −42 of the *hET-1* 5′-flanking sequence.

ACCCCC Is the Minimal Binding Site within the Vezf1/DB1 Response Element—In an effort to define the exact nucleotides necessary for Vezf1/DB1 binding within the *hET-1* 5′-flanking sequence, we made a series of 2- and 4-bp mutations in the minimal putative binding motif within the 3′ probe (Mut-1 to Mut-7, Fig. 6*A*) and tested their ability to compete for binding with the wild-type radiolabeled 3′ probe. Mutants 2, 3, and 4 failed to compete for this binding activity (Fig. 6*B*), whereas mutants 1, 5, 6, and 7 competed with efficiency nearly equal to that of the wild-type probe, indicating that ACCCCC is the minimal sequence required to recapitulate the binding activity observed in endothelial cell nuclear extracts.

Vezf1/DB1 Interacts Directly with the Minimal Binding Element in the Vezf1/DB1 Response Element—If Vezf1/DB1 *trans*-activates the *hET-1* promoter by direct interactions with sequences within the *hET-1* 5′-flanking sequence, we can then hypothesize that one or more of these specific binding activities contains endogenous Vezf1/DB1. To explore this hypothesis, we



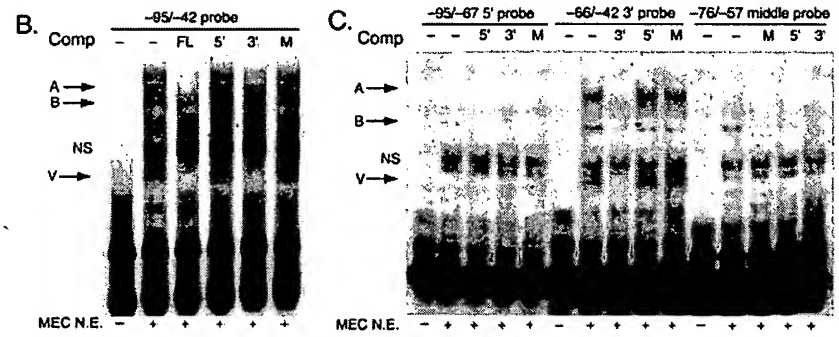


FIG. 4. **Determination of a Vezf1/DB1 response element within the human endothelin-1 5′-flanking sequence.** The indicated luciferase reporter constructs were transiently transfected into MEC with (*black bars*) or without (*white bars*) 0.5 μg of a Vezf1/DB1 expression vector. The presence of defined response elements relative to the reporter constructs used is indicated on the *left*. The vector pCMV-βGal was used to correct for differences in transfection efficiency. Luciferase activity is expressed as fold induction relative to transfection in the absence of Vezf1/DB1. The results are expressed as the mean ± S.E.

FIG. 5. **Identification of nuclear protein interactions within the Vezf1/DB1 response element of the human endothelin-1 5′-flanking sequence.** *A*, overlapping probes within the Vezf1/DB1 response element used to characterize nuclear protein interactions. *B*, EMSA was performed with a ^{32}P-labeled oligonucleotide corresponding to bp −95 to −42 of the *hET-1* 5′-flanking sequence. Addition of nuclear extracts (*N.E.*) from MEC resulted in three specific retarded DNA-protein complexes (labeled *A*, *B*, and *V*) and a nonspecific (*NS*) activity. Specific complexes were competed by a molar excess cold full-length (*FL*) competitor (*Comp*) and by excess cold 3′ competitor, but not by the 5′ or middle (*M*) competitors. *C*, EMSAs were performed with either the 5′, 3′, or middle probes, as indicated, with or without MEC nuclear extracts and the indicated competitors. Specific binding complexes *A*, *B*, and *V* were only observed to form with the 3′ probe.

tested the ability of recombinant Vezf1/DB1 (expressed in bacteria as a GST fusion) to bind to the minimal binding element by EMSA (Fig. 6*C*). This experiment shows that recombinant Vezf1/DB1 can, indeed, form a DNA-protein complex with the 3′ probe, which can be competed away with an excess of the wild-type unlabeled probe. In addition, the binding determinants for binding with recombinant Vezf1/DB1 were identical to those of nuclear extract, based on competition experiments with the mutated oligonucleotides. These experiments indicate that Vezf1/DB1 can indeed bind directly to a specific sequence within the *hET-1* 5′-flanking sequence; in addition, they argue strongly that the binding activity of nuclear extract consists, in part, of endogenous Vezf1/DB1, and provide a rationale for examining whether ACCCCC is the bona fide Vezf1/DB1 response element.

ACCCCC Is the Vezf1/DB1 Response Element in the hET-1 5′-Flanking Sequence—To test the hypothesis that the sequence ACCCCC is indeed the Vezf1/DB1 response element, we mutated 2 bp in this sequence (analogous to Mut-3 in the EMSA experiments, Fig. 6*A*) in the context of the wild-type *ET-1* promoter and tested the ability of Vezf1/DB1 to *trans*-activate this mutated promoter in transient transfection assays. In contrast to the wild-type *ET-1* promoter, the mutated promoter was resistant to *trans*-activation by Vezf1/DB1 (Fig. 7, *A* and *B*). Thus, we can conclude that the minimal sequence ACCCCC is indeed the Vezf1/DB1 response element in the human *ET-1* promoter.

Vezf1/DB1 Is Present in DNA-Protein Complexes Formed in Nuclear Extracts from Endothelial Cells—We typically see more than one specific complexes by EMSA with the ACCCCC element (Figs. 5 and 6, *arrows*). The similarity between the binding determinants for nuclear extracts and recombinant Vezf1/DB1 indicates that one or more of these activities is likely to contain Vezf1/DB1 itself. The diversity of the binding activities in endothelial nuclear extracts may reflect one or more of the following: (i) multiprotein complexes containing Vezf1/DB1; (ii) additional nuclear proteins binding the same element; or (iii) proteolytic products producing different gel

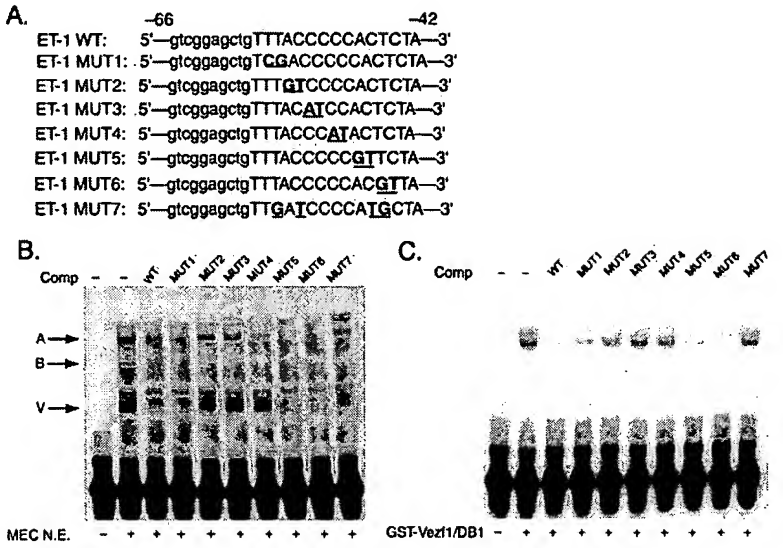


FIG. 6. **ACCCCC is a Vezf1/DB1-binding site within the human endothelin-1 5′-flanking sequence.** *A*, oligonucleotides used as competitors in EMSA experiments. Residues that differ from the wild-type sequence are *underlined*. *B*, EMSAs were performed with MEC nuclear extract (*N.E.*) as indicated using the 3′ probe. The indicated unlabeled 3′ oligonucleotides containing 2- or 4-bp mutations were used as competitors (*Comp*). Failure of MUT2, MUT3, and MUT4 constructs to compete for formation of specific complexes indicates that these residues are critical for nuclear protein interactions. *C*, similar EMSAs with recombinant GST-Vezf1/DB1 demonstrate specific binding of the fusion protein within the Vezf1/DB1 response element, and an identical pattern of competition in comparison with nuclear extract.

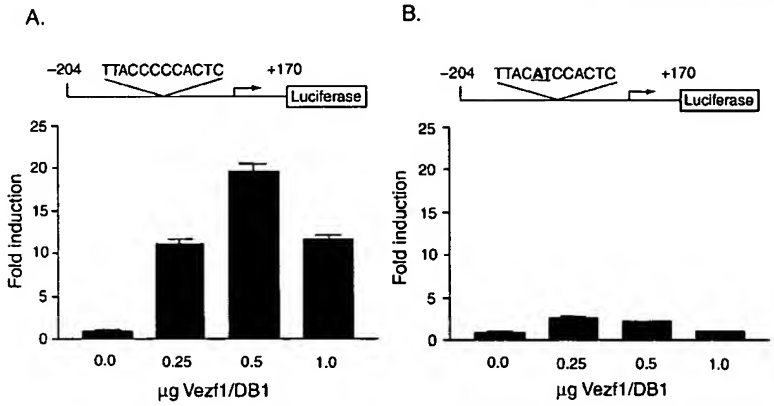


FIG. 7. **ACCCCC is the minimal Vezf1/DB1 response element in the human endothelin-1 5′-flanking sequence.** Luciferase reporter construct pGL2–204/+170 with a wild-type (*A*) or mutated (*B*) Vezf1/DB1 response element was transiently transfected into MEC with the indicated concentrations of the Vezf1/DB1 expression vector. The vector pCMV-βGal was used to correct for differences in transfection efficiency. The results are expressed as the mean ± S.E.

shift patterns. To discern among these possibilities, we have raised affinity purified rabbit polyclonal antibodies against a 15-amino acid Vezf1/DB1 peptide (MEANWTAFLFQAHEC). As a more precise test of the component of DNA-protein complex, we preincubated nuclear extracts from MEC with anti-Vezf1/DB1 antibody prior to the EMSA binding reactions. Formation of specific DNA-protein complex *A* was attenuated, and a supershifted band (*SS*) appeared, when nuclear extracts were preincubated with the specific antibody, whereas a similarly prepared nonspecific antibody did not have any effect on the binding pattern (Fig. 8*A*).

The supershift experiment strongly suggested that Vezf1/DB1 was present in MEC nuclear extracts and participated in nuclear protein complex formation with the human *ET-1* promoter. As additional confirmation that Vezf1/DB1 bound specifically to the *ET-1* 5′-flanking sequence, we examined the interaction of purified Vezf1/DB1 to the ET-1 primer (Fig. 8*B*). Under the conditions employed, a stable complex could form when recombinant GST-Vezf1/DB1 (but not GST alone) was used in these binding assays. As before, these complexes could be disrupted by an excess of specific cold oligonucleotide, but not by a similar concentration of a nonspecific competitor. This complex could be supershifted with an anti-GST antibody but not by a nonspecific antibody, indicating that these complexes were formed with the fusion protein, and not by a co-purifying activity. In addition, these complexes could also be abolished by the specific anti-Vezf1/DB1 antibody (*data not shown*). These experiments demonstrate that nuclear extracts from MEC contain Vezf1/DB1 protein and that Vezf1/DB1 is involved in a specific DNA-protein complex with the *hET-1* 5′-flanking sequence that contains the Vezf1/DB1 response element.

Vezf1/DB1 Trans-activates the hET-1 Promoter Maximally in Endothelial Cells—Because the expression of Vezf1/DB1 is restricted to the vascular endothelium (14), we wanted to determine whether its forced expression was sufficient to *trans*-activate the *ET-1* promoter in non-endothelial cell types. To test this, we transiently transfected MEC or non-endothelial cell lines (C2C12 and NIH/3T3) with either the wild-type or mutated *hET-1* promoter constructs and the Vezf1/DB1 expression plasmid. Consistent with our previous results, Vezf1/DB1 potently *trans*-activated the *hET-1* promoter by over 20-fold, whereas a 2-bp mutation in the Vezf1/DB1 response element abolished Vezf1/DB1 responsiveness in MEC (Fig. 9). The *hET-1* promoter was also *trans*-activated by Vezf1/DB1 in C2C12 and NIH/3T3 cells; however, the *trans*-activation was only 4- and 5-fold in C2C12 and NIH/3T3 cells, respectively. This *trans*-activation was dependent on the same Vezf1/DB1 response element that is functional in MEC (compare wild-type and mutated promoters). These results indicate that Vezf1/DB1 is sufficient to *trans*-activate the *hET-1* promoter in both endothelial and non-endothelial cells when it is expressed ectopically. It is likely that the differences in *trans*-activation among cell types reflect the presence of additional factors in endothelial cells that are required for maximal activation of the *hET-1* promoter by Vezf1/DB1. This interpretation is consistent with the presence of multiple DNA-protein complexes seen in our EMSA experiments (Figs. 5, 6, and 8).

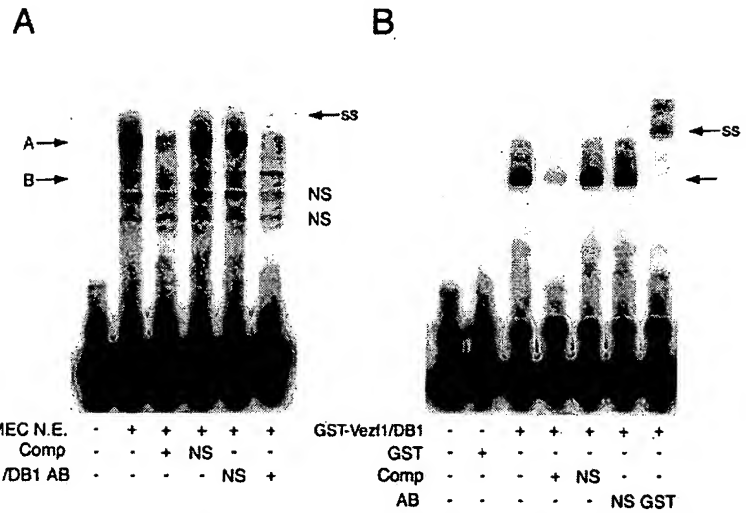


FIG. 8. **Identification of Vezf1/DB1 in nuclear protein complexes with the human endothelin-1 5′-flanking sequence.** *A*, EMSAs performed with a double stranded, ^{32}P-labeled oligonucleotide corresponding to bp −66 to −42 identified specific complexes *A* and *B*, as determined by competition experiments with specific (+) and nonspecific (*NS*) competitors (*Comp*). Preincubation of the nuclear extract-DNA complex with affinity purified Vezf1/DB1 antibody competed away the higher molecular weight complex *A*, which was supershifted (*SS*), whereas a nonspecific antibody had no effect on formation of complex *A*. *B*, similar EMSAs using the labeled oligonucleotide corresponding to bp −66 to −42 and GST-Vezf1/DB1 or GST alone in a binding assay demonstrate a single specific complex (*arrow*) that was formed between the probe and GST-Vezf1/DB1 (but not with GST alone), which was competed by specific, but not nonspecific competitors, and that could be supershifted (*SS*) with an anti-GST antibody, but not by a nonspecific antibody.

DISCUSSION

In this report, we identify Vezf1/DB1 as an endothelial cell-specific transcription factor. We also characterize a logical transcriptional target, and the DNA response element through which this target is chosen. Vezf1/DB1 potently *trans*-activates the hET-1 5′-flanking sequence through a novel response element, ACCCCC, to mediate high level transcriptional activity of this promoter. Proteins from endothelial cell nuclear extracts bind specifically to this essential response element, and endogenous Vezf1/DB1 exists as a component of this binding activity. These results provide convincing evidence that Vezf1/DB1 acts as a transcription factor and that *ET-1* is a transcriptional target for this protein. In addition, these studies provide a rational explanation for inducible expression of ET-1 specifically within the vascular endothelium.

Vezf1/DB1 was first identified by screening an expression library with a GC-rich Tax-responsive element within the interleukin-3 promoter (15). Recombinant Vezf1/DB1 could bind to this GC-rich sequence by EMSA; however, Vezf1/DB1 by itself was not able to *trans*-activate the interleukin-3 promoter, although it did modulate Tax-mediated *trans*-activation in a phorbol ester-dependent fashion. We have similarly seen low-affinity interactions between Vezf1/DB1 and GC-rich regions within the KDR/flk-1 promoter in EMSA experiments,[2] yet Vezf1/DB1 does not *trans*-activate the KDR/flk-1 promoter (Fig. 2*A*). Therefore, we suspect these low affinity interactions with GC-rich sequences may not be physiologically significant. In contrast, the highly specific interactions identified between Vezf1/DB1 and the ACCCCC motif result in potent *trans*-activation of the *ET-1* promoter. These observations, in conjunction with the overlapping expression pattern of Vezf1/DB1 and ET-1 during development (4, 14), makes regulation of *ET-1* by Vezf1/DB1 a more plausible physiologic interaction to regulate gene expression in vascular endothelial cells. The ACCCCC sequence characterized in these studies as the Vezf1/DB1-binding site and response element does not exactly correspond to any previously identified transcription factor-binding sites, as determined by searches of the TRANSFAC data base. However, it is interesting to note that MAZ, which is closely related to Vezf1/DB1 and also contains 6 highly similar zinc fingers of the Cys_2/His_2 type, binds to response elements containing stretches of G or C residues (23, 24), suggesting that this family of transcription factors may have particularly high affinity for homopolymeric stretches of G/C residues. Identification of a defined Vezf1/DB1-binding site will aid in the discovery of other Vezf1/DB1-responsive genes within the vascular endothelium.

The studies presented here do not specifically address how Vezf1/DB1 activity itself is regulated, and what the consequences of differences in Vezf1/DB1 activity might mean with respect to ET-1 expression, although some interesting hypotheses can be generated based on our observations and those of others. ET-1 expression in endothelial cells is known to be dependent on Rho GTPase activity, and Rho signaling itself can directly activate the *ET-1* promoter (25). This effect may have particular importance with respect to modulating vasoconstrictive, migratory, and proliferative effects of cells that are ET-1-responsive, such as smooth muscle and neural crest cells, especially since Rho signaling pathways down-regulate endothelial nitric-oxide synthase (26), which opposes the actions of ET-1 within the vasculature. The relationship between ET-1 expression and Rho signaling is significant because physical interactions have been demonstrated between Vezf1/DB1 and the fraction of prenylated RhoB that is localized to the nucleus (27). It is tempting to speculate that Vezf1/DB1 serves, at least in part, to mediate Rho-dependent signaling events, such as ET-1 expression, at the transcriptional level in endothelial cells.

Although previous data provide few insights into the function of Vezf1/DB1, the expression of this gene during development argues strongly for a specific role in vascular development. The expression of Vezf1/DB1 overlaps significantly during development with KDR/flk-1 (14), a receptor for vascular endothelial growth factor and a marker for endothelial cells and their precursors during development in the mouse (28). This would suggest: 1) that Vezf1/DB1 may lie upstream of

[2] J. Aitsebaomo and C. Patterson, unpublished observations.

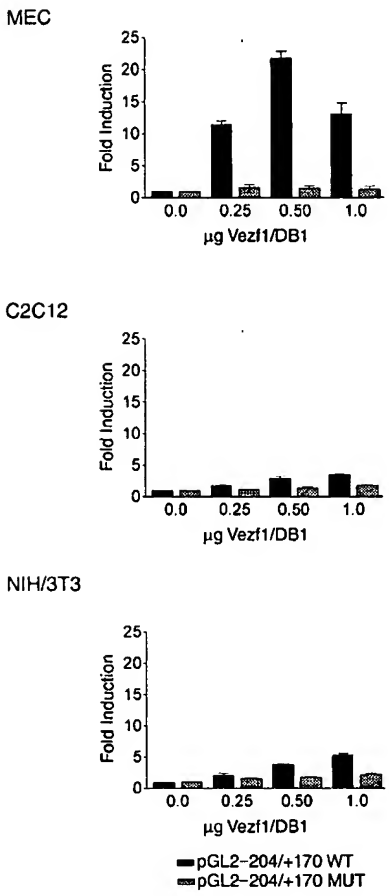


FIG. 9. **Vezf1/DB1 transactivation of the *hET-1* promoter in endothelial and non-endothelial cells.** The luciferase reporter construct pGL2–204/+170, either with a wild-type (*WT*) or mutated (*MUT*) Vezf1/DB1 response element, was transiently transfected into MEC, NIH/3T3, or C2C12 cells with the indicated concentrations of the Vezf1/DB1 expression vector. The vector pCMV-βGal was used to correct for differences in transfection efficiency. The results are expressed as the mean ± S.E.

KDR/flk-1 by up-regulating its expression; 2) that Vezf1/DB1 is induced in KDR/flk-1-expressing cells; or 3) that KDR/flk-1 and Vezf1/DB1 are regulated in parallel, possibly through similar mechanisms. We have found no evidence for the first possibility, that Vezf1/DB1 regulates expression of KDR/flk-1[2] (Fig. 2*A*). Instead, we find that Vezf1/DB1 *trans*-activates *ET-1*, which appears later than KDR/flk-1 during vascular development. Deficiency of ET-1 during development results in branchial arch abnormalities, leading to defects in branchial arch artery development and subsequent malformation of the great vessels and cardiac outflow tract abnormalities, as well as characteristic craniofacial abnormalities (4, 5). If the effects of Vezf1/DB1 on ET-1 expression observed in the present studies are representative of regulatory events that are critical in endothelial cells during vascular development, then absence of Vezf1/DB1 in mice should phenocopy, at least partially, *ET-1*-null mice. Experiments to delete Vezf1/DB1 in mice by homologous recombination will determine whether the molecular events described in the present studies are essential for the regulation of *ET-1* during development.

Although cell type-specific gene regulation is beginning to be understood for many lineages, such as skeletal myocytes (29) and smooth muscle cells (30), there is still little known about the transcriptional mechanisms that regulate cell type-specific gene expression in the vascular endothelium. For example, KDR/flk-1 is a well characterized marker for vascular endothelial cells during development, and studies have demonstrated the importance of a variety of transcription factors in control of its expression, including Sp1 (31), TFII-I (21), and GATA proteins (32, 33). However, none of these proteins by themselves account for the restricted pattern of KDR/flk-1 expression during development. Similarly, GATA-2, AP-1, and HIF-1 have all been implicated in the transcriptional regulation of ET-1 (9, 10, 12), yet the means by which ET-1 expression is induced specifically within the vascular endothelium is still not explained.

Several reports have implicated Ets family members in endothelial development and cell type-specific gene regulation (34, 35); however, most of these proteins are expressed in many lineages other than endothelial cells, so Ets proteins are likely required to cooperate with factors that are expressed in a more restricted fashion to mediate endothelial cell gene regulation. The helix-loop-helix transcription factor SCL/tal-1 has been implicated in endothelium-specific transcriptional events as well. Although SCL/tal-1 is dispensable for endothelial cell specification but required for generation of all hematopoietic lineages (36–38), a role in endothelial pattern formation has been attributed to SCL/tal-1 (39). These data would suggest that SCL/tal-1 functions to direct endothelial cell gene expression in early stages of development, although this conclusion has been drawn into question by the demonstration that these effects of SCL/tal-1 are DNA-binding independent (40). In any event, endothelial transcriptional targets for SCL/tal-1 have not been well characterized, and the prominent role of SCL/tal-1 in hematopoietic transcriptional events indicates that SCL/tal-1 cannot, by itself, explain endothelial cell type-specific gene expression. In contrast, the identification of Vezf1/DB1 as a developmentally regulated, endothelial specific protein (14), and our demonstration here that it is a nuclear localizing protein that functions as a transcriptional activator, indicates that Vezf1/DB1 may serve as an important missing link in our understanding of endothelial cell type-specific gene regulation. Although further studies will be necessary to determine the range of endothelial cell genes that are regulated by Vezf1/DB1, and the functional role of this protein in the modulation of endothelial cell phenotypes, our studies support the hypothesis that Vezf1/DB1, in cooperation with other transcription factors yet to be determined, assists in the cellular process of determining the complement of genes that are expressed within the vascular endothelium.

Acknowledgment—We thank Victoria Bautch for editorial comments.

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Vezf1 Function is Required for Murine Vascular Development and Haploinsufficiency Results in Lymphatic Hypervascularization

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Short title: Vascular and lymphatic defects in *Vezf1* KO mice

Key words: *Vezf1* function, ES cells, knockout mice, endothelial cells, vascular development, angiogenesis, lymphangiogenesis

Total word count: 7,807 (including 1,186 in figure legends)

SUMMARY

Vezf1 is an early development gene that encodes a zinc finger transcription factor. In the developing embryo, *Vezf1* is expressed in the yolk sac mesoderm and the endothelium of the developing vasculature and, in addition, in mesodermal and neuronal tissues. Targeted inactivation of *Vezf1* in mice reveals that it acts in a tightly regulated, dose-dependent fashion on the development of the blood vascular and lymphatic system. Our studies suggest that at the cellular level *Vezf1* functions in endothelial cells where it affects cell proliferation and integrity of the vessels. Homozygous mutant *Vezf1* embryos display angiogenic remodeling defects and loss of vascular integrity. Furthermore, in heterozygous embryos, lymphatic hypervascularization is observed that is associated with hemorrhaging and edema localized in the jugular region. This haploinsufficient phenotype is reminiscent of a human congenital malformation syndrome of the lymphatic system, cystic hygroma.

INTRODUCTION

Mammals have two structurally different circulatory systems, the closed blood vasculature and the open lymphatic system, that are functionally connected and act in concert to maintain tissue homeostasis. The blood vascular system, consisting of arteries, capillaries and veins, efficiently carries nutrients, gases and waste products to and from distant actively metabolizing tissues. The lymphatic system regulates tissue fluid balance by returning interstitial fluid and macromolecules from the tissue spaces of most organs back into the venous circulation and serves as a conduit for trafficking immune cells, thus complementing the function of the blood vascular system.

During embryonic development the blood vascular system is formed through two distinct processes, vasculogenesis and angiogenesis (Risau, 1997; Carmeliet, 2000). Vasculogenesis describes the initial differentiation of mesodermally-derived endothelial precursor cells, angioblasts, and their coalescence into a primitive vascular network. Angiogenesis refers to the subsequent growth, remodeling and maturation processes of the primary vascular plexus that ultimately give rise to the mature blood vasculature. The development of the lymphatic system, lymphangiogenesis, has been the center of longstanding controversy dating back to the beginning of the twentieth century (Huntington, 1908; Sabin, 1909). The recent identification of markers specific for lymphatic endothelial cells such as the homeobox transcription factor Prox1 (Wigle and Oliver, 1999), the hyaluronan receptor LYVE-1 (Banerji et al., 1999), secondary lymphoid chemokine (SLC) (Gunn et al., 1998) and vascular endothelial growth factor receptor 3 (VEGFR-3) (Kaipainen et al., 1995) has triggered the reevaluation of this process, lending support to the theory of a venous origin of the lymphatics and proposing a stepwise model of lymphatic specification (Wigle et al., 2002). Thus, the appearance of a subpopulation of LYVE-1- and Prox1-expressing endothelial cells in the anterior cardinal vein around embryonic day 10 marks the onset of lymphatic differentiation. This subpopulation of endothelial cells buds and migrates from the cardinal vein in a polarized manner to give rise to the jugular lymph sacs.

Capillary plexi spread from the jugular sacs to the neck, head, forelimbs, and thorax. The remaining lymphatic sacs (retroperitoneal, posterior and cisterna chili) originate from the mesonephric vein and veins in the dorsomedial edge of the Wolffian bodies, and they develop subsequent to the formation of the jugular lymph sacs (Gray, 1985).

The vascular endothelial growth factor (VEGF) signaling pathway plays a critical role in the regulation of both blood vascular and lymphatic development. VEGF-A signaling, through binding to its blood endothelial cell-specific receptors VEGFR-1 and VEGFR-2, is essential for the early stages of blood vascular development and the initiation of vascular sprouting (Carmeliet et al., 1996a; Ferrara et al., 1996). Similarly, overexpression of VEGF-A in the skin of transgenic mice stimulates growth of blood vascular capillaries without affecting lymphatic vessels (Detmar et al., 1998; Thurston et al., 1999). In contrast, selective activation of VEGFR-3 by receptor-specific mutants of VEGF-C and VEGF-D induces lymphangiogenesis in the same transgenic mouse model (Veikkola et al., 2001). The importance of VEGFR-3 signaling for lymphatic development is underscored by the fact that overexpression of soluble VEGFR-3 leads to inhibition of lymphangiogenesis (Makinen et al., 2001), and that mutations in the tyrosine kinase domain of VEGFR-3 have been linked to human hereditary primary lymphedema (Karkkainen et al., 2000). Angiopoietin signaling, in addition to its critical function during blood vascular remodeling and vessel stabilization (Gale and Yancopoulos, 1999), has recently been shown to be a regulator of lymphatic development. Thus, inactivation of angiopoietin-2 leads to defects in postnatal patterning and function of the lymphatic vasculature (Gale et al., 2002).

At the level of transcriptional regulation, Prox1 activity is required for maintaining lymphatic endothelial cell sprouting and loss of Prox1 function results in arrested lymphatic development without affecting blood vessel formation (Wigle et al., 2002; Wigle and Oliver, 1999). Moreover, misexpression of Prox1 in blood endothelial cells confers a lymphatic endothelial phenotype, indicating that Prox1 is a master regulator of the lymphatic endothelial

cell fate (Hong et al., 2002; Petrova et al., 2002). Using gene inactivation approaches, several transcription factors have been implicated in blood vascular development (for review see (Oettgen, 2001). For instance, the genetic ablation of the bHLH-PAS protein hypoxia inducible factor 1α (HIF-1α) leads to defective yolk sac and cephalic vascularization (Iyer et al., 1998; Ryan et al., 1998), while the zinc finger lung Kr ppel-like factor (LKLF) is required for vascular smooth muscle cell and pericyte recruitment during vessel stabilization (Kuo et al., 1997). However, none of these transcription factors appears to play a role during lymphatic development.

Vascular endothelial zinc finger 1 (*Vezf1*) was originally identified as a gene specifically expressed in vascular endothelial cells during early embryonic development, although our subsequent analysis indicated expression in mesodermal and neuronal tissues as well [Xiong, 1999 #328; F. Kuhnert and H. Stuhlmann, unpublished]. *Vezf1* encodes a 518 amino acid nuclear protein that contains six zinc finger motifs of the C2H2 (Kr ppel-like)-type and a proline-rich transcriptional transactivation domain at its C-terminus (Sun et al., 2003). Consistent with the hypothesis that VEZF1 is a bona fide transcription factor, the human ortholog ZNF161/DB1 has been shown to selectively transactivate the endothelial cell-specific human endothelin-1 promoter in vitro (Aitsebaomo et al., 2001). To investigate the role of *Vezf1* in vivo, we have generated a null allele by gene targeting. Here we report that inactivation of *Vezf1* results in angiogenic remodeling defects and loss of vascular integrity in homozygous mutant embryos. Furthermore, loss of a single *Vezf1* allele leads to an incompletely penetrant phenotype characterized by lymphatic hypervascularization that is associated with hemorrhaging and edema in the jugular region. This haploinsufficient phenotype is reminiscent of the human congenital malformation syndrome, cystic hygroma (Gallagher et al., 1999). Our studies demonstrate that *Vezf1* is a crucial regulator of blood vessel and lymphatic development that acts in a tightly regulated dose-dependent fashion during embryonic development.

MATERIALS AND METHODS

Construction of the Vezf1 targeting construct and ES cell manipulations

A 5 *Vezf1* cDNA fragment (nt 3-776 of the published mouse *Vezf1* cDNA sequence (Xiong et al., 1999) GeneBank accession # AF104410, was used as probe to screen a mouse 129/Sv lambda genomic library (provided by K. Andrikopoulous and F. Ramirez, Mount Sinai School of Medicine). One positive phage clone contained the first and second exon separated by 5-kb of intronic sequence, and 15-kb of genomic sequence upstream of exon 1. For construction of the targeting vector, an 8-kb EcoRV/XhoI fragment containing the first exon, 6-kb of upstream genomic sequences and 2-kb of 3 intronic sequence was subcloned into pBluescriptIISK (Stratagene). A 390-bp EagI fragment including the ATG translation initiation codon was replaced with an IRES-*lacZ*gt1.2*neo* cassette (Wang and Lufkin, 2000) via NotI linkers. Subsequent analysis of the *Vezf1* promoter revealed that the deletion included 91-bp upstream of the transcriptional start site, resulting in a non-functional *lacZ* allele.

R1 ES cells (Nagy et al., 1993) were maintained in DMEM (high glucose) containing 15% heat-inactivated fetal calf serum (FCS), 0.1 mM β-mercaptoethanol, 20 mM HEPES, pH 7.3, 0.1 mM non-essential amino acids, and 1000 u/ml LIF on γ-irradiated primary MEFs as a feeder layer. The targeting vector was linearized with XhoI and introduced into R1 cells by electroporation (400V, 125 μF; BioRad Gene Pulser). ES cell clones were selected at G418 concentrations of 200-350 μg/ml for 10 days. ES cell clones homozygous for the targeted *Vezf1* allele were obtained by hyperselection (Mortensen et al., 1992) at concentrations between 400-1000 μg/ml of G418 for 14 days. Clones with correctly targeted alleles were identified by Southern blot analysis of BamHI-digested genomic DNA, using a [α-^{32}P]-dCTP-labeled 5 genomic fragment (EcoRV-EcoRV fragment of 1-kb length), and a 3 cDNA fragment corresponding to the second exon (BamHI-PstI of 500-bp length) as probes. ES cells were

induced to differentiate in suspension cultures into embryoid bodies as described previously (Leahy et al., 1999).

Northern analysis

Total RNA was prepared from ES cells and embryoid bodies using a RNeasy Mini Kit (Qiagen). RNA (10 μg each) was analyzed on Northern blots as described (Xiong et al., 1999), using a [α-^{32}P]-dCTP-labeled *Vezf1* cDNA fragment (nucleotides 3-776) as a probe. To control for RNA loading, filters were stripped and hybridized with [α-^{32}P]-dCTP-labeled mouse GAPDH cDNA plasmid.

Generation of Vezf1 KO mice and genotyping

Chimeric mice were generated by injection of *Vezf1*$^{+/-}$ ES cells into C57BL/6 blastocysts (performed by the Transgenic Core Facility at Mount Sinai School of Medicine). Mice at weaning age and staged embryos from E14.5 to birth (the morning of the vaginal plug was counted as day-0.5 post coitum) were genotyped by Southern blot analysis of BamHI-digested tail or yolk sac DNA, respectively. A 500-bp fragment from exon 2 was used as a 3 flanking probe for Southern blot analysis (see above). Staged embryos from E8.5 to E13.5 were genotyped by genomic PCR. Primers for the wild type *Vezf1* allele are: 5 (5 -GCGTCCCGGAGGTTACCGAAGTGG-3) and 3 (5 -GGAACAGGAACGCGGTCCAGTTGG-3), yielding an amplification product of 150-bp; primers for the targeted allele are: 5 (5 -TTTCTCTGGGCCGCGGGGTGGT-3) and 3 (5 -GCGGGGATCTCATGCTGGAGTT-3), yielding a 300-bp amplification product.

Whole-mount PECAM-1 staining of embryos and yolk sacs

Staged embryos from the fifth backcross generation (F5) were fixed in 4% paraformaldehyde (PFA)/PBS overnight at +4°C, dehydrated through a methanol series and stored in 100% methanol at -20°C. Embryos were bleached in 6% hydrogen peroxide/methanol for 1 hour at room temperature and rehydrated through a methanol series to PBS/0.1% Tween 20 (PBST). After blocking in 3% instant skim milk/PBST (PBSMT) twice for 1 hour, the embryos were incubated overnight at +4°C with a monoclonal rat anti-mouse CD31 (PECAM-1) antibody (1:50 dilution in PBMST; MEC13.3, Pharmingen) (Krebs et al., 2000). Embryos were washed with PBSMT five times for 1 hour at +4°C and incubated with peroxidase-conjugated donkey anti-rat secondary antibody (Jackson Immunoresearch) overnight at +4°C in PBST. Peroxidase reaction was visualized with DAB/hydrogen peroxide. Yolk sacs were embedded in paraffin, thin-sectioned and counterstained with Nuclear Fast Red.

Histology and immunohistochemistry

F5 litters from timed (*Vezf1*$^{+/-}$ x *Vezf1*$^{+/-}$) matings were recovered between E8.5 and birth. Yolk sacs were isolated for genotyping and embryos were photographed in ice-cold PBS under a Nikon SMZ-U stereomicroscope with a 35mm camera. After overnight fixation in 4% PFA/PBS at +4°C, the embryos were dehydrated and embedded in paraffin. Serial sections (5-μm) were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). Antigen retrieval was performed by incubation for 30 min at 95°C in Target Retrieval Solution (DAKO). Endogenous peroxidase activity was quenched by incubation in 5% hydrogen peroxide/methanol for 5 min. Sections were incubated with horseradish peroxidase-conjugated anti-SMα-actin antibody (1:100 dilution, EPOS anti-SMαA/HRP, DAKO) for 1 hour at room temperature. After washing with PBS, peroxidase reaction was visualized with diaminobenzidine (DAB)/hydrogen peroxide. For PECAM-1/CD31 staining, sections were pretreated for antigen

retrieval with Proteinase K (36 μg/ml) for 30 min at 37°C, and incubated overnight at +4°C with rat anti-mouse CD31 antibody (1:100 dilution, Pharmingen). Samples were washed and incubated with a biotinylated anti-rat secondary antibody, and peroxidase reaction was visualized using a tyramide amplification system (NEN). VEGFR-3 staining was accomplished by overnight incubation at +4°C with monoclonal rat-anti-mouse VEGFR-3 antibody (kindly provided by H. Kubo and K. Aritalo) as described (Kubo et al., 2000). Samples were washed, incubated with biotinylated anti-rat secondary antibody, and peroxidase reaction was visualized using the tyramide amplification system. For LYVE-1 staining, embryo sections were pretreated with 0.25 mg/ml trypsin in 0.05M Tris, pH 7.8. Sections were incubated overnight at +4°C with polyclonal rabbit-anti-mouse LYVE-1 antibody (kindly provided by D. Jackson) (Prevo et al., 2001). Samples were washed, incubated with biotinylated anti-rabbit secondary antibody, and peroxidase reaction was visualized using the tyramide amplification system. All antibody-stained sections were counterstained with hematoxylin. Photomicrographs were taken under a Zeiss Axioplan2 microscope with a digital Zeiss AxioCam color camera.

For immunohistochemical staining using Prox1 antibodies (kindly provided by G. Oliver), staged embryos were fixed overnight at 4°C in 4% PFA/PBS, cryoprotected in 30% sucrose/PBS, and embedded in OCT (TissueTek). Ten-micron cryostat sections were post-fixed for 15 min in 4% PFA/PBS and incubated for 10 min at 37°C in 10 μg/ml Proteinase K/0.2 M Tris-HCl, pH 7.2. To block unspecific binding, slides were incubated for 30 min at room temperature in TNB buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% NEN blocking solution). Primary antibodies (rat anti-mouse CD31 at 2.5 g/ml; rabbit anti-Prox1, 1:100 dilution) were incubated overnight at 4°C, and staining was revealed with Alexa488-labelled rabbit anti-rat IgG and Cy3-labelled goat anti-rabbit IgG, respectively. Control sections were incubated with nonspecific rabbit and rat IgG using conditions as described above. Slides were mounted with

fluorescent mounting medium (DAKO), examined under epifluorescence (Zeiss Axioplan2) and photographed.

Vessel morphometry

PECAM-1 stained sections of E13.5 wild type and heterozygous mutant embryos were counterstained with hematoxylin. Sections were imaged with a digital camera (Pixera, Los Gatos, CA). Images were processed by using Scion Image software (Frederick, MD). PECAM-1 positive structures in the neck region of embryos were automatically counted, and their area was measured (values represent area occupied by PECAM-1 positive structures per microscopic field, expressed in percent). Differences in vascularity (number of and area occupied by CD31-positive structures per embryo microscopic field) were determined for each section. Endothelial linear density was determined by quantitating the number of endothelial cells (nuclei of PECAM-1 positive cells), per vessel wall unit. A total of 3 heterozygous mutant and wild type embryos each were analyzed.

Cultivation of megakaryocytes from embryonic livers

Fetal livers from E12.5 embryos were isolated and placed into low-glucose DMEM supplemented with 10% FCS and 10 ng/ml mouse TPO. Liver cells were dissociated by drawing them subsequently through 20g and 25g needles. Cells from one liver were seeded into a 6-well dish and incubated at 37°C in 5% CO_2. After 6 days of culture, cells were visually examined under the microscope, harvested and stained with Wright-Giemsa solution.

Gene expression analysis

Total RNA was isolated from E10.5 embryos using Trizol (Life Technologies) and treated with DNaseI (Life Technologies) according to the manufacturer s instructions. Total RNA (2 μg) was reverse transcribed using random hexamers with the Superscript First Strand Synthesis

System (Life Technologies) according to the manufacturer s instructions. PCR was performed using 1-µl of the reverse transcriptase reaction in a volume of 50-µl using Hotstar Taq polymerase (Qiagen). The PCR conditions were as follows: initial denaturation at 95°C for 10 min followed by up to 35 cycles of denaturation at 95°C (1 min), annealing at 58°C (1 min), and extension at 72°C (1 min). 5-µl aliquots were taken after 25, 30 and 35 cycles and amplified PCR products were analyzed by electrophoresis on a 2% agarose gel. PCR primer pairs were as follows:

Flk-1:	5 -TCTGTGGTTCTGCGTGGAGA-3 ,
	5 -GTATCATTTCCAACCACCCT-3 ;
Flt-1:	5 -TGTGGAGAAACTTGGTGACCT-3 ,
	5 -TGGAGAACAGCAGGACTCCTT-3 ;
Flt-4:	5 -CACCGAAGCAGACGCTGATGAT-3
	5 -AGCTGCTGTCTGCGAAGAAG-3 ;
VEGF-A:	5 -GTAACGATGAAGCCCTGGAGTG-3
	5 -TGAGAGGTCTGGTTCCCGAAAC-3 ;
VEGF-C:	5- AAATGTACAAGTGCCAGCTGCGGAA-3
	5 -GGCATCGGCACATGTAGTTATTCCA-3
VEGF-D:	5 -ACGATCTGAACAACAGATCCGAGCA-3
	5 -TCTGGGGTCTGAATGGATCTTCTGA-3
Tie1:	5 -TCTTTGCTGCTCCCCACTCT-3
	5- ACACACCATTCGCCATCAT-3 ;
Tie2:	5 -CCTTCCTACCTGCTACTTTA-3
	5 -CCACTACACCTTTCTTTACA-3 ;
Ang1:	5 -AAGGGAGGAAAAAGAGAAGAAGAG-3 ,
	5 -GTTAGCATGAGAGCGCAT TTG-3 ;
Ang2:	5 -TGCCTACACTACCAGAAGAAC-3 ,
	5 -TATTTACTGCTGAACTCCCAC-3 ;
Notch1:	5 -TGCCTGAATGGAGGTAGGTGCGAA-3 ,
	5 -GCACAGCGATAGGAGCC GATCTCA-3 ;
Notch4:	5 -CCAAGAGATTCCCTTAAACTCGG-3 ,
	5 -CCAGAGTTTAGGGATTCTC G-3 ;

Dll4:	5 -GACTGAGCTACTCTTACCGGGTCA-3 ,
	5 -CTTACAGCTGCCACCATTTC GACA-3 ;
EphrinB2:	5 -CTGTGCCAGACCAGACCAAGA-3 ,
	5 -CAGCAGAACTTGCATCTTG TC-3 ;
EphB4:	5 -CAGGTGGTCAGCGCTCTGGAC-3 ,
	5 -ATCTGCCACGGTGGTGAGTC- 3 ;
Tbx1:	5 -GTTGCAGCCTTCGCAGCCAGCA-3 ,
	5 -TAGTGTACTCGGCCAGGTGTA GCA-3 ;
LKLF:	5 -CCACACATACTTGCAGCTACAC-3 ,
	5 -CCATCGTCTCCCTTATAGAAAT A-3 ;
EDG-1:	5 -TAGCAGCTATGGTGTCCACTAG-3 ,
	5 -GATCCTGCAGTAGAGGATGG C-3 ;
Endoglin:	5 -TACTCATGTCCCTGATCCAGCC-3 ,
	5 -GTCGATGCACTGTACCTTTTT CC-3 ;
VE-cadherin:	5 -GGATGCAGAGGCTCACAGAG-3 ,
	5 -CTGGCGGTTCACGTTGGACT-3 ;
ET-1:	5 -TGTCTTGGGAGCCGAACTCA-3 ,
	5 -GCTCGGTTGTGCGTCAACT TCTGG-3 ;
Vezf1:	5 -GTCTCATGAAGGAGGCATCACCA-3 ,
	5 -ACATGTTTTACATGACAGCT TAGGT-3 ;
β-actin :	5 - GTGGGCCGCTCTAGGCACCAA -3 ,
	5 - CTCTTTGATGTCACGCACGATTTC -3 .

RESULTS

Targeted disruption of the Vezf1 gene in mouse embryonic stem cells

To disrupt the *Vezf1* gene in mouse ES cells, a 20-kb genomic *Vezf1* fragment was isolated that contained the first and second coding exons of 153 and 695 bp length, respectively, the first intron of 5-kb length, and 15-kb of genomic sequence upstream of exon 1 (Fig.1A, top). A targeting vector was constructed that replaced exon 1 and the proximal part of the first intron with a promoter-less IRES-*lacZ* gene and a *neo* expression cassette (Fig.1A). The construct was introduced into R1 ES cells (Nagy et al., 1993), and three independent clones (#2, 74, 86) out of 96 G418-resistant clones analyzed contained the correctly targeted allele (Fig.1B). An ES cell clone that carried the introduced mutation in both alleles (Fig.1C) was obtained by G418 hyperselection (Mortensen et al., 1992). Using a probe corresponding to the second coding exon, low levels of *Vezf1* transcripts were detected by Northern analysis in undifferentiated wild type R1 cells, and high levels in day-6 embryoid bodies derived from wild type R1 cells (Fig.1D), consistent with previous studies (Xiong et al., 1999). In contrast, *Vezf1* mRNA was reduced by about half in embryoid bodies derived from heterozygous ES cells and was absent in undifferentiated $Vezf1^{-/-}$ ES cells or in embryoid bodies derived from this clone (Fig.1D). Furthermore, *Vezf1* mRNA could not be detected in KO embryos by RT-PCR using primer pairs spanning the entire coding sequence (data not shown), indicating that the targeting event resulted in a null mutation. All three independently derived ES cell clones transmitted the targeted allele through the germ line.

Disruption of Vezf1 results in vascular defects and lethality in homozygous and heterozygous embryos

The *Vezf1* mutation was examined in the C57BL/6 background. For this, F5 offspring from $Vezf1^{+/-}$ intercrosses were analyzed for viability. Heterozygous $Vezf1^{+/-}$ mice were viable, fertile, and appeared phenotypically indistinguishable from their wild type littermates. In contrast,

no viable *Vezf1*$^{-/-}$ offspring were obtained (Table 1). Embryonic death and intraembryonic hemorrhage of the *Vezf1* KO phenotype became apparent at E9.5 (Table 1, Fig.2A). Hemorrhaging or resorbed *Vezf1*$^{-/-}$ embryos at sub-Mendelian ratios were found up to E16.5 (Table 1, Fig.4A). Interestingly, beginning at E12.5, 20% of heterozygous embryos displayed localized edema and hemorrhaging in the back and neck (Table 1, Fig.5A). At E13.5, these mutant *Vezf1*$^{+/-}$ embryos appeared bloated, displaying massive edema, hemorrhaging, and signs of tissue necrosis in the back and neck (Fig.5C). Hemorrhaging *Vezf1*$^{+/-}$ embryos were not detected at later embryonic stages and presumably died before E14.5.

In a mixed and outbred genetic background, a less severe and less penetrant phenotype was observed. Heterozygous F1 (129/Sv x C57BL/6) males were bred with outbred CD-1 females, and offspring from F2 intercrosses were analyzed (Table 2). Whereas no viable *Vezf1*$^{-/-}$ offspring were recovered at weaning age, stillborn P0 *Vezf1*$^{-/-}$ pups displaying intraembryonic hemorrhage were detected at a ratio of ~60% of the expected Mendelian frequency. *Vezf1*$^{-/-}$ embryos appeared phenotypically normal up to E11.5 of development. Between E12.5 and birth, *Vezf1* KO embryos displayed varying degrees of intraembryonic bleeding and vascular malformations in the head and trunk region, or they were resorbed (data not shown). Vascular abnormalities were also observed in heterozygous mutant embryos, albeit at a low frequency (~10%).

In summary, loss of *Vezf1* function resulted in an incompletely penetrant, haploinsufficient (autosomal) phenotype, which was not caused by imprinting of the *Vezf1* gene or by generation of a dominant negative allele. This result indicates that *Vezf1* acts in a tightly regulated, dose-dependent manner during embryonic development. Interestingly, the mutant phenotype in the C57BL/6 background was more severe and less variable than that in a mixed genetic background, suggesting contribution of yet unknown strain-specific, segregating modifier genes.

Vascular remodeling defects and hemorrhaging in E9.5 Vezf1$^{-/-}$ embryos

The developing vasculature was examined in detail in E9.5 *Vezf1* KO embryos and yolk sacs, at the onset of mutant phenotypes, by whole-mount immunostaining for the pan-endothelial marker PECAM-1 (Baldwin et al., 1994). PECAM-1-stained wild type (Fig.2E) and *Vezf1*$^{+/-}$ E9.5 littermates (not shown) exhibited a complex, hierarchically organized vessel architecture. In the trunk region the following vascular structures are easily recognizable: the dorsal aorta, the first three aortic arch arteries, the anterior cardinal vein, and the intersomitic vessels, which branch dorsally into an arborized capillary network that extends anteriorly. The head shows large caliber vessels branching off the anterior cardinal vein and the internal carotid artery to branch into a finer vascular lattice. *Vezf1*$^{-/-}$ mutant embryos had normally formed dorsal aortae, cardinal veins and most major vessels, however they displayed prominent abnormalities in the aortic arch artery system (Fig.2 A,B). A functional second aortic arch artery was missing in all embryos examined (compare Fig.2B and E), and half of these embryos displayed a rudimentary first branchial arch artery that had failed to develop a lumen. In addition, the vasculature in the neck region and the dorsal part of the intersomitic vessels showed poor organization and was less branched than in their wild type littermates (compare Fig.2B and E). Although the head vasculature in *Vezf1*$^{-/-}$ mutants consisted of large and small caliber vessels, it appeared disorganized and less developed when compared to wild type controls (Fig.2B). Histological analysis of parasagittal sections through PECAM-1 stained E9.5 *Vezf1*$^{-/-}$ embryos confirmed the absence of the second and malformations of the first aortic arch artery (compare Fig.2C and F). In addition, histological analysis revealed distinct sites of internal bleeding, typically in the head and trunk. Thus, in the embryo shown (Fig. 2A,C), the pericardial cavity was enlarged and filled with blood, and an unusual number of dilated, hemorrhaging vessels was apparent in the head.

In contrast to the observed abnormalities in the embryo proper, no vascular defects were detected in the extra-embryonic yolk sac, the initial site of endothelial and hematopoietic differentiation. Whole-mount PECAM-1-stained E9.5 yolk sacs from mutant embryos showed a

hierarchical vascular pattern that was indistinguishable from that of wild type controls (compare Fig.3A and B), consistent with normal vasculogenesis and remodeling. Furthermore, PECAM-1-stained sections of mutant yolk sacs displayed normally developed blood islands with an outer layer of endothelial cells surrounding lumens that are filled with primitive, nucleated erythrocytes, and regular vascular patterning without any signs of lesions or hemorrhaging (compare Fig.3C and D). Taken together, these results indicate that *Vezf1* function is neither required for the formation of the primary vascular plexus (vasculogenesis) nor for primitive hematopoiesis in the yolk sac, but is critically important for angiogenic remodeling and the maintenance of vascular integrity in the embryo proper.

Localized hemorrhaging in Vezf1$^{-/-}$ embryos between E12.5 and E16.5

A small fraction of homozygous mutant embryos survived to day-16 of gestation, and gross examination showed localized hemorrhaging (Table 1, Fig.4A). To analyze development of the vascular system in KO embryos at late midgestation stages, parasagittal sections of paraffin-embedded mutant *Vezf1$^{-/-}$* embryos were stained with anti-PECAM-1 antibody. Mutant E12.5 *Vezf1$^{-/-}$* embryos displayed apparently normal vascular patterning that was indistinguishable from that observed in age-matched wild type littermates (Fig.4E,F), demonstrating overall normal angiogenic remodeling in these embryos. In addition, development of all organ systems, including heart, lung and placenta appeared normal in H&E stained histological sections (Fig.4C,D). However, localized hemorrhaging, typically in the head and in the neck region, was observed in these embryos, indicating the loss of vascular integrity. The representative E12.5 *Vezf1$^{-/-}$* embryo shown in Fig.4C displays hemorrhaging in the subcapsular mesenchyme of the head and in the jugular region.

Normal vascular smooth muscle cell recruitment and megakaryocyte development in Vezf1 KO embryos

To further characterize the underlying cause for the loss of vascular integrity in *Vezf1* KO embryos on a cellular level, we investigated vascular smooth muscle cell (VSMC) and megakaryocyte development in mutant embryos.

Defects in the differentiation and/or recruitment of VSMC or pericytes result in destabilized, leaky blood vessels and lead to lethal hemorrhage, as has been shown previously in mice lacking Edg-1, LKLF and PDGF-B (Liu et al., 2000; Kuo et al., 1997; Lindahl et al., 1997). To investigate a possible involvement of VSMC in the hemorrhaging observed in mutant E12.5 *Vezf1*$^{-/-}$ embryos, parasagittal sections of embryos shown in Fig.4 were immunostained with anti-SMα-actin antibodies. In sections of *Vezf1*$^{-/-}$ and wild type control embryos, the dorsal aorta was completely surrounded by VSMCs (Fig.4G,H). Likewise, cross sections through the dorsal aorta (Fig.4I,J) and umbilical artery (not shown) of mutant embryos showed several layers of VSMC. Thus, mural cell defects can be excluded as a cause for the vascular defects in E12.5 *Vezf1*$^{-/-}$ embryos.

Likewise, defects in the differentiation of megakaryocytes lead to defective platelet formation and control of coagulation, resulting ultimately in lethal hemorrhaging (Spyropoulos et al., 2000). To address whether megakaryocytopoiesis was affected in *Vezf1* KO embryos, wild type and *Vezf1*$^{-/-}$ E12.5 fetal liver cells were cultured and assayed *in vitro* for their differentiation potential into megakaryocytes (Era et al., 1997). Normal megakaryocyte and proplatelet differentiation was apparent in day-5 *in vitro* cultures and Wright-Giemsa-stained cytospin preparations derived from homozygous mutant fetal liver cultures (Fig.4K,L), indicating that *Vezf1* is not required for the proper development of the megakaryocyte lineage. Therefore, we conclude that loss of vascular integrity and intraembryonic bleeding in *Vezf1* KO embryos is likely to be a direct result of deficiencies of the vascular endothelium.

Vascular malformations and hemorrhaging in E13.5 embryos heterozygous for the targeted Vezf1 allele

Beginning at E12.5 hemorrhaging and edema, confined mostly to the jugular region, became apparent in about 20% of *Vezf1*$^{+/-}$ embryos (Table 1; compare Fig.5 A and B). By E13.5, embryos appeared bloated and displayed massive edema and bleeding in the back and neck region (compare Fig.5C and D). H&E-stained parasagittal sections of heterozygous E13.5 embryos showed severe edema, with masses of loose connective tissue and mesenchymal cells separated by interstitial fluid, and subcutaneous bleeding in the back and neck region (Fig.5E,I). Furthermore, a dramatic increase in the vessel number was detected in close association with the jugular sac in *Vezf1*$^{+/-}$ embryos (Fig.5E,I) when compared to wild type controls (Fig.5F,J). PECAM-1-staining revealed that *Vezf1*$^{+/-}$ embryos displayed an overall normal looking vascular pattern, and that hypervascularization appeared confined to the jugular region (Fig.5, compare panels G,K,O to panels H,L,P). Vessels in the jugular region appeared enlarged, irregular shaped, and anastomosing, and they were occasionally filled with blood (Fig.5K,M). Under high-power magnification, sites of vascular lesions were visible with erythrocytes leaking out into the surrounding mesenchymal tissue (Fig.5N), indicating loss of vessel integrity. Quantitative morphometric analysis of PECAM-1 stained sections confirmed that the overall vessel number and the total vessel area were increased significantly in the mid-back region of mutant heterozygous embryos (Fig.6A,B). In addition, the endothelial cell density (i.e. the number of endothelial cells per vessel wall unit) was significantly higher in the jugular region of the embryo than in wild type control embryos (Fig.6C). Together, these results suggest that localized endothelial hyperproliferation in the jugular region leads to the formation of aberrant, dysfunctional vessels in heterozygous embryos.

Lymphatic hypervascularization in E13.5 Vezf1$^{+/-}$ mutant embryos

Hypervascularization in mutant *Vezf1*$^{+/-}$ embryos was observed specifically in the jugular region which constitutes the first major site of lymphatic development (Sabin, 1909). Thus, we analyzed lymphatic vessel formation in E13.5 wild type and *Vezf1*$^{+/-}$ embryos by immunostaining for the lymphatic markers VEGFR-3 (Kaipainen et al., 1995), LYVE-1 (Banerji et al., 1999), and Prox1 (Wigle and Oliver, 1999). In wild type embryos, expression of VEGFR-3 (Fig.7B,H) and LYVE-1 (Fig.7D,J) was detected in the endothelium lining the jugular sac and in small capillaries of the neck mesenchyme. In *Vezf1*$^{+/-}$ embryos, in addition to these structures, the hyperplastic vessels in the jugular region stained positive for these two lymphatic markers (VEGFR-3: Fig.7A,G; LYVE-1: Fig.7C,I). Double immunofluorescence staining for PECAM-1 and Prox1 revealed that the majority of endothelial cells in the jugular region of heterozygous embryos appear disorganized and have lymphatic characteristics (Fig.7K,L), whereas in wild type controls only a subset of small capillaries are of lymphatic nature (Fig.7M,N). In contrast, the hyperplastic vessels did not express SMα-actin, a marker for blood vessel-associated VSMCs (Fig.7E,F). In summary, our results indicate that the hyperplastic vessels in *Vezf1*$^{+/-}$ embryos are of lymphatic nature and that loss of a single *Vezf1* allele leads to lymphatic hypervascularization in the jugular region. This haploinsufficient phenotype is reminiscent of a human malformation syndrome, cystic hygroma, a congenital malformation of the lymphatic system that occurs most commonly in the posterior neck and may be caused by abnormal budding of lymphatic endothelium (Edwards and Graham, 1990; Gallagher et al., 1999). Moreover, the fact that the hyperplastic lymphatic vessels contain blood suggests deficient separation of the emerging lymphatic vessels from the blood vasculature in the heterozygous *Vezf1* mice (Abtahian et al., 2003).

Gene expression analysis

Our results indicate that loss of *Vezf1* function leads to defects in endothelial cell proliferation, angiogenic remodeling, and maintenance of vascular integrity. A number of factors have been implicated in these processes and shown to display similar or partially overlapping mutant phenotypes in gene inactivation studies (Carmeliet, 2000). To assess if loss of *Vezf1* function could be correlated with changes in expression levels for any of these factors, semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis was performed on total RNA isolated from E10.5 *Vezf1* $^{-/-}$ embryos and wild type littermates. Because of the incompletely penetrant phenotype, E10.5 *Vezf1* KO embryos were selected that displayed clear signs of hemorrhaging and vascular malformations. Expression of the following groups of genes was examined (Fig.8): (1) VEGF-A, -C and -D, and VEGFR-1, -2 and —3. (2)*Angiopoietin-1* and *-2,* their cognate tyrosine kinase receptor *Tie2*, and the closely related orphan receptor *Tie1* (Gale and Yancopoulos, 1999). (3) *Notch1*, *Notch4* and its ligand *Dll4* that have been implicated in vascular morphogenesis (Krebs et al., 2000). (4) *EphrinB2* and its receptor *EphB4* that demarcate embryonic arterial versus venous endothelium and are involved in vascular remodeling (Adams et al., 1999; Krebs et al., 2000; Wang et al., 1998). (5) *LKLF*, a Kr ppel-like zinc finger transcription factor, *Edg-1*, the G-protein-coupled receptor for sphingosine-1-phosphate, and *Endoglin*, an accessory TGFβ-receptor, all of which show hemorrhaging KO phenotypes (Kuo et al., 1997; Li et al., 1999; Liu et al., 2000). (6) *Tbx-1*, a T-box transcription factor required for development of the aortic arch artery system in a gene-dosage dependent manner (Lindsay et al., 2001). (7) *VE-cadherin*, an endothelial cell adhesion molecule and regulator of endothelial cell survival (Carmeliet et al., 1999). (8) *Endothelin-1* (ET-1), a vasoconstrictive signaling peptide. ET-1 inactivation leads to malformations in the aortic arch system (Kurihara et al., 1994), and the human ET-1 promoter is transactivated by ZNF161/DB1 in vitro (Aitsebaomo et al., 2001). As expected, *Vezf1* transcripts were absent in E10.5 *Vezf1*$^{-/-}$ embryos and present in wild type littermates. In contrast, no differences in the expression of any

of the candidate genes examined were found between mutant and wild type embryos (Fig.8). In addition, the distribution of transcripts for *Flk-1, Flt-1, EphrinB2, Tie2* and ET-1 was analyzed by RNA in situ hybridization on E11.5 embryos. No differences in the amount or spatial distribution of these transcripts between *Vezf1*$^{-/-}$ and wild type embryos were detected (data not shown). Thus, *Vezf1* function is not required for the expression of these candidate genes known to regulate mammalian vasculogenesis and angiogenesis.

DISCUSSION

We report here that *Vezf1* is essential for normal development of the vascular system. Inactivation of *Vezf1* leads to embryonic lethality, and mutant embryos display specific defects during angiogenic remodeling, vascular homeostasis and lymphatic development. Analysis of the homozygous mutant phenotype revealed that, while its function is dispensable for the early stages of vascular development, namely angioblast differentiation and the formation of the primary vascular plexus, *Vezf1* is required for proper angiogenic remodeling and the maintenance of vascular integrity. Homozygous mutant embryos can be subdivided into two classes. Class 1 embryos display an early phenotype that is characterized by defective angiogenic remodeling at E9.5, particularly in the developing vasculature of the aortic arch system, the head and neck, and in the intersomitic vessels, and by the loss of vascular integrity leading to hemorrhaging. In contrast, class 2 embryos display normal vascular patterning but are subject to midgestation hemorrhaging. The manifestation of vascular leakage in these later stage KO embryos without concomitant vascular dismorphogenesis suggests that hemorrhaging in class 1 embryos is independent of the angiogenic remodeling defects. This notion is further supported by the fact that similar remodeling defects were reported for the inactivation of *Angiopoietin1*, *Tie2*, *VEGFR-3*, *ephrinB2*, *EphB4*, *EphB2/B3* and *Notch1/Notch4*, and that these mutants did not display any vascular leakage (Adams et al., 1999; Dumont et al., 1998; Gerety

et al., 1999; Krebs et al., 2000; Sato et al., 1995; Suri et al., 1996; Wang et al., 1998). Interestingly, in those studies abnormal vascular remodeling was also observed in the extraembryonic yolk sac, whereas the yolk sac vasculature appeared normal in *Vezf1* KO embryos.

Our studies strongly suggest that at the cellular level the primary defect has its origin in the vascular endothelial cells themselves. First, *Vezf1* is expressed in endothelial cells during embryogenesis (Xiong et al., 1999). Second, breaching of the capillary endothelium could be demonstrated in hemorrhaging embryos. Third, two alternative causes were addressed in this study, namely defects in VSMC differentiation and/or recruitment (Kuo et al., 1997; Liu et al., 2000) and defective megakaryocyte differentiation (Spyropoulos et al., 2000). However, both processes were found to be normal in homozygous mutant embryos. Thus, we surmise that the underlying defect is either loss of structural integrity of the endothelial cells (Sato et al., 1995), defective inter-endothelial cell contacts, or defective interactions between endothelial cells and the underlying basal membrane (Vestweber, 2000). In this context it is of interest to note that members of the blood coagulation system, such as tissue factor and PAR1, have been implicated in the regulation of endothelial cell function during blood vessel formation and vascular remodeling (Bugge et al., 1996; Carmeliet et al., 1996; Griffin et al., 2001). We are presently testing if forced transgenic expression of *Vezf1* under Tie2 promoter control in endothelial cells can rescue the mutant phenotype in null embryos (F. Kuhnert, Z. Zou, and H. Stuhlmann, unpublished).

The elucidation of the mechanism of *Vezf1* function on the molecular level is limited by the fact that, thus far, no in vivo downstream target genes have been identified. In this study, the expression of a large canon of genes known to regulate endothelial cell proliferation, angiogenic remodeling and the maintenance of vascular integrity was examined. Loss of *Vezf1* function could not be correlated with changes in the expression levels for any of these genes. In a recent study, in vitro binding and transactivation of the human ET-1 promoter by ZNF161/DB1, the

human ortholog of *Vezf1*, was demonstrated (Aitsebaomo et al., 2001). Interestingly, mice deficient for ET-1 display defects in the aortic arch artery system (Kurihara et al., 1995; Kurihara et al., 1994). However, no effects of *Vezf1* inactivation on ET-1 transcript levels and its spatial distribution could be detected. A micorarray expression profiling approach (Brown and Botstein, 1999) should be instrumental in identifying *Vezf1* target genes and thus provide insights into the molecular mechanisms that underlie *Vezf1* function. Taken together, the specific defects observed in *Vezf1* mutant embryos and the expression profile of candidate target genes suggest that *Vezf1* may function through yet unknown pathways to regulate vascular development.

Our results indicate that *Vezf1* acts in a tightly regulated, dose-dependent manner during vascular development. Approximately 20% of heterozygous embryos displayed hyper-vascularization associated with edema and hemorrhaging in the jugular region. This incompletely penetrant, haploinsufficient (autosomal) phenotype is caused neither by imprinting of the residual wild type allele, nor by the generation of a dominant-negative VEZF1 mutant peptide. Only few examples of haploinsufficiency in genes crucial for vascular development have been reported; these include VEGF-A and TGF-β (Carmeliet et al., 1996a; Dickson et al., 1995; Ferrara et al., 1996). In addition, the T-box transcription factor *Tbx-1* is required specifically for the development of the pharyngeal arch arteries in a concentration-dependent manner (Lindsay et al., 2001).

Our results indicate that *Vezf1* is involved in the regulation of early events of lymphangiogenesis. First, histological and quantitative morphometric analysis of heterozygous mutant embryos at E13.5 demonstrated an increased number of enlarged, irregular shaped, anastomosing, leaky vessels in the jugular region, the first site of lymphatic development (Sabin, 1909). The increased total vessel numbers and the increased linear endothelial density suggest endothelial hyperproliferation as a plausible mechanism for the observed vascular hyperplasia. Second, the hyperplastic vessels express the lymphatic markers VEGFR-3 (Kaipainen et al., 1995), LYVE-1 (Banerji et al., 1999), and Prox1 (Wigle and Oiver, 1999). Together, our data

indicate that loss of a single *Vezf1* allele leads to lymphatic hypervascularization in the nuchal region, thus identifying *Vezf1* as a negative regulator of lymphatic development. The lymphatic nature of the hyperplastic vessels appears to conflict with the fact that many of these contain red blood cells. However, it has been reported that lymphatic capillaries may contain stagnant blood, which is removed into the venous circulation once the lymphatic system becomes functional (Clark, 1912; Lewis, 1905; Miller, 1913). Alternatively, separation of the emerging lymphatic vessels from the blood vasculature may be defective in the mutant mice, similar to the phenotype recently reported for the inactivation of the hematopoietic signaling proteins SLP-76 and Syk (Abtahian et al., 2003).

Lymphatic development is initiated by budding and sprouting of Prox1 and LYVE-1 positive cells from the anterior cardinal vein at around E10 (Oliver and Harvey, 2002). The nascent lymphatic endothelial cells migrate dorsoanteriorly to form the jugular lymph sacs. Prox1 activity is required for maintaining sprouting of lymphatic endothelial cells, and loss of Prox1 function results in the arrest of lymphatic development (Wigle et al., 2002; Wigle and Oliver, 1999). Moreover, misexpression of Prox1 in blood endothelial cells confers a lymphatic endothelial phenotype, indicating that Prox1 is a critical regulator of the lymphatic endothelial cell fate (Hong et al., 2002; Petrova et al., 2002). Careful etiological analysis will be necessary to further elucidate the mechanism underlying the lymphatic phenotype in *Vezf1*$^{+/-}$ embryos. For example, it will be important to examine if overexpression of *Vezf1* in blood endothelial cells antagonizes Prox1 function, and if forced *Vezf1* expression in lymphatic endothelial cells has anti-proliferative effects.

The lymphatic phenotype observed in E13.5 *Vezf1*$^{+/-}$ embryos is reminiscent of a human malformation syndrome, cystic hygroma. Cystic hygromas are congenital malformations of the lymphatic system that occur at sites of lymphatic-venous connection, most commonly in the posterior neck (Gallagher et al., 1999). Typically, cystic hygromas develop late in the first trimester, either as a consequence of failure of the lymphatic vessels to connect to the venous

system, leading to the accumulation of fluid in dilated lymphatics and to progressive lymphedema, or due to abnormal budding of lymphatic endothelium (Edwards and Graham, 1990). Rates as high as 1 in 100 unselected pregnancies have been reported for this syndrome (Gallagher et al., 1999). Although cystic hygromas are frequently associated with other malformation syndromes characterized by chromosomal abnormalities, such as Turner syndrome or Trisomy 21, fetal nuchal cystic hygromas with normal karyotypes have been reported as well (Marchese et al., 1985). It will be important to examine if haploinsufficiency of the human ortholog ZNF161/DB1 is associated with this human syndrome. Moreover, it will be interesting to determine if loss of ZNF161/DB1 function can be correlated with other known hyperplastic conditions thought to arise by excessive proliferation of lymphatic endothelial cells, such as lymphangioma, lymphangiosarcoma, and Kaposi s sarcoma (Witte et al., 1997).

In summary, our results identify *Vezf1* as a gene whose function is crucial for the normal development of blood and lymphatic vasculature in mice in a dosage-dependent manner. At the cellular level, *Vezf1* acts in the endothelial cells themselves and affects proliferation and integrity of the developing vessels. *Vezf1* might participate in yet unknown pathways that affect vascular differentiation and endothelial proliferation. To elucidate these molecular pathways, it will be important to identify genes that function in the same transcriptional regulatory circuits as VEZF1.

ACKNOWLEDGEMENTS

We would like to acknowledge Dr. Nissi Varki (UC San Diego School of Medicine) to consult with us on the pathology of the mutant *Vezf1* mice, and Dr. Kevin Kelly (Mount Sinai School of Medicine Transgenic Core) for blastocyst injections. We thank Wendy LeVine for expert technical assistance with mouse husbandry, histology and immunohistochemistry. We are thankful to Drs. K. Andrikopoulous and F. Ramirez (Mount Sinai School of Medicine, New York) for the mouse genomic 129/SV lambda library; and Drs. H. Kubo and K. Aritalo, D. Jackson, G. Oliver for providing us with antibodies to mouse VEGFR-3, LYVE-1, and Prox1, respectively. We thank Dr. Ullrich M ller and members of the Stuhlmann lab for critical comments on the manuscript, and Kathy Kling for help with preparing the manuscript. This work was supported by NIH grants R29 HD31534 and R01 HL65738, and Grant-in-Aid #99505N from the American Heart Association-National Center, to H.S. J.-W.X. was supported in part by a NIH training grant (T32 DK07757).

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Table 1.

Genotype of embryos from *Vezf1*$^{+/-}$ intercrosses in the F5 C57BL/6 backcross generation[a].

DEVELOPM. STAGE	# OF EMBRYOS	+/+	+/-*	-/-*
E 8.5	9	2	6	1
E 9.5	60	18	33	9 (5)
E10.5	33	8	21	4 (2)
E11.5	30	6	21	3 (1)
E12.5	56	14	36 (7)	6 (2)
E13.5	14	1	12 (3)	1
E14.5	13	5	7	1
E16.5	11	2	8	1 (1)
P0	21	9	13	0

[a] Embryos were genotyped by performing PCR on genomic yolk sac DNA with primer pairs specific for the wild type and targeted allele.

* Numbers in parentheses represent embryos with vascular abnormalities and hemorrhaging

Table 2.

Genotype of progeny from heterozygous *Vezf1*$^{+/-}$ intercrosses in a mixed genetic background[a].

DEVELOPM. STAGE	# OF EMBRYOS/PUPS	+/+	+/-	-/-
E10.5	14	3	7	4
E11.5	14	3	6	5
E12.5	44 2 resorbed	16 0	26 0	2 2
E13.5	10	3	7	0
E14.5	35 1 resorbed	10 0	20 0	5 1
E15.5	12 2 resorbed	2 0	9 0	1 2
E16.5	2	1	1	0
P0	53	15	30	8 (stillborn)
P21	46	16	30	0

[a] Embryos were genotyped by performing PCR on genomic yolk sac DNA with primer pairs specific for the wild type and targeted allele.

FIGURE LEGENDS

Figure 1. Targeted inactivation of the *Vezf1* gene

(A) Targeting strategy. Exons are represented as numbered boxes. The first exon, 91 bp of upstream genomic DNA and the proximal part of the first intron were replaced with an IRES-*lacZ-neo* expression cassette by homologous recombination in R1 ES cells. Regions of homology between the endogenous locus (top) and the targeting vector (middle) are indicated by lines. The structure of the targeted allele is shown on the bottom. Locations of probes for Southern analysis corresponding to 5 and 3 flanking regions are indicated by bars. B, BamHI; E, EagI; E5, EcoRV; N, NotI; X, XhoI. **(B)** Southern blot analysis of three correctly targeted ES cell clones. 10-μg genomic DNA was digested with BamHI and hybridized with the 5 flanking probe shown in (A). Sizes of hybridizing fragments are indicated. Genotypes of ES cell clones are indicated at the top of each lane. Three independent targeted *Vezf1*$^{+/-}$ ES cells clones (#2, 74, 86) were identified. **(C)** Southern blot analysis of DNA isolated from individual ES cell clones subjected G418 hyperselection protocol. Experimental procedures are identical to those in (B). One *Vezf1*$^{-/-}$ clone (lane 2) was recovered. **(D)** Northern blot analysis of total RNA from ES cells and day-6 embryoid bodies using a *Vezf1* cDNA fragment (nucleotides 3-776) as a probe. The filter was reprobed with a GAPDH-specific probe to control for loading. Genotypes are indicated at the top of each lane. No *Vezf1*-specific transcript was detected in *Vezf1*$^{-/-}$ ES cells and embryoid bodies.

Figure 2. Vascular defects in E9.5 *Vezf1*$^{-/-}$ embryos.

(A,D) Photomicrographs of E9.5 *Vezf1* KO (-/-) and wild type (+/+) embryos. *Vezf1*$^{-/-}$ embryos displayed hemorrhaging in the head, the trunk and the pericardial cavity. **(B,E)** Whole mount immunohistochemistry for PECAM-1. The vasculature in head, neck and dorsal part of intersomitic vessels of *Vezf1*$^{-/-}$ embryos (B) appeared disorganized and less developed than in wild type controls (E). The arrow indicates absence of the second aortic arch artery in the *Vezf1*$^{-/-}$

embryo. **(C,F)** Histological sections of same E9.5 embryos. *Vezf1*$^{-/-}$ embryo (C) displayed hemorrhaging in the head and the pericardial cavity (arrowheads). Note also the absence of the second aortic arch artery (arrow). (F) Wild type control.

Figure 3. Normal vascular development in *Vezf1*$^{-/-}$ yolk sacs.

(A,B) Photomicrographs of whole-mount PECAM-1-stained E9.5 yolk sacs. *Vezf1*$^{-/-}$ yolk sacs (A) displayed a regular hierarchical vascular pattern that is indistinguishable from that of wild type controls (B). **(C,D)** Sections of the same PECAM-1-stained yolk sacs were counterstained with Nuclear Fast Red. Inserts, details of yolk sacs at high magnification. Blood islands are lined by PECAM-1-postive endothelial cells and filled with primitive, nucleated erythrocytes (arrows). *Vezf1*$^{-/-}$ yolk sacs (C) did not show any signs of vascular disorganization or lesion, (D) Wild type control.

Figure 4. Hemorrhaging in E12.5 *Vezf1*$^{-/-}$ embryos.

(A,B) Whole-mount images of E12.5 *Vezf1*$^{-/-}$ (A) and wild type (B) embryos. The E12.5 KO embryo displayed hemorrhaging in the jugular region and subcapsular head mesenchyme (arrows). Parasagittal sections shown in C, E, G are derived from the *Vezf1* KO embryo depicted in (A), and sections shown in D, F, H are from the wild type embryo depicted in (B). **(C,D)** Histological analysis of H&E-stained E12.5 *Vezf1*$^{-/-}$ (C) and wild type (D) embryo. Arrows in (C) indicate sites of bleeding in the jugular region and the subcapsular head mesenchyme. **(E,F)** Immunohistochemical PECAM-1 staining revealed overall normal vascular patterning in *Vezf1*$^{-/-}$ (E) and wild type (F) embryos. **(G,H)** Immunohistochemical SMα-actin staining demonstrated normal VSMC differentiation and recruitment in *Vezf1* KO (G) and wild type (H) embryos. **(I,J)** In transverse sections of anti-SMα-actin stained embryos, the dorsal aorta is surrounded by several layers of VSMCs in *Vezf1* KO (I) and wild type embryos (J). **(K, L)** Whright-Giemsa staining of day-5 *in vitro* cultures of E12.5 fetal liver cells shows normal

megakaryopoiesis in *Vezf1*$^{-/-}$ embryos (K) as compared to a wild type control embryo (L). Arrows indicate examples of megakaryocytes.

Figure 5. Hypervascularization, edema, and hemorrhaging in heterozygous *Vezf1*$^{+/-}$ embryos.

(A-D) Photomicrographs of whole-mount E12.5 (A, B) and E13.5 (C,D) embryos. Hemorrhaging became apparent in the jugular region of a mutant E12.5 *Vezf1*$^{+/-}$ embryo (A). Affected E13.5 *Vezf1*$^{+/-}$ embryo (C) displayed severe edema and hemorrhaging in the back and neck. (B,D) Wild type controls. **(E,F)** H&E staining of parasagittal sections of E13.5 *Vezf1*$^{+/-}$ (E) and wild type control (F) embryos. Severe edema in the back region, hypervascularization and hemorrhaging are visible in the jugular area of the heterozygous embryo (E). Boxed areas depict regions shown at higher magnification in panels I and J. **(G,H)** PECAM-1 staining of parasagittal sections close-by to those shown in (E,F). PECAM-1 staining outlines the embryonic vasculature and shows that hypervascularization is confined to the neck and back region of the mutant embryo (G). Boxed areas depict regions shown under higher magnification in panels K and O, and L and P, respectively. **(I,J)** High magnification images of the jugular region from embryos shown in (E) and (F). Arrowheads: abnormal vessels in jugular region; * jugular sac. **(K-N)** High magnification photomicrographs of the jugular region of mutant *Vezf1*$^{+/-}$ embryo (K,M,N) and a wild type control (L) shown in (G) and (H). Hypervascularization with abnormal vessel morphology and loose surrounding connective tissue is visible in the jugular region (K,M). Lesions in the endothelial wall of small vessels and erythrocytes leaking into the surrounding connective tissue (arrow) are detected in the neck region (N). **(O,P)** High magnification photomicrographs of the dorsal region of mutant Vezf1$^{+/-}$ embryo (O) and wild type control (P) shown in (G) and (H). The heterozygous mutant embryo (O) displayed tissue swelling and masses of loose connective tissue.

Figure 6. Quantitative morphometric analysis of the vasculature in the jugular region of heterozygous and wildtype E13.5 embryos.

(A) Total vessel number; (B) Total vessel area; (C) Endothelial linear density; (n=3).

Figure 7. Lymphatic hypervascularization in heterozygous mutant E13.5 embryos.

(A-F) Parasagittal sections of an E13.5 *Vezf1*$^{+/-}$ embryo (A,C,E) and wild type control embryo (B,D,F). Sections were immunostained for the lymphatic markers VEGFR-3 (A,B) and LYVE-1 (C,D), and for the VSMC marker SMα-actin (E,F). The hyperplastic vessels in the jugular region of heterozygous E13.5 embryos stain positive for the lymphatic markers VEGFR-3 (A) and LYVE-1 (C), but are negative for SMα-actin (E). **(G,H)** High magnification of the jugular region from images shown in (A,B). **(I,J)** High magnification of the jugular region from images shown in (C,D). Jugular sacs in wild type control are depicted by *. Examples of hyperplastic vessels in heterozygous embryos are depicted by arrows in (G,I). **(K-N)** Cryostat sections from a mutant *Vezf1*$^{+/-}$ (K,L) embryo and a wild type littermate (M,N) were double immunostained for PECAM-1 (K,M) and Prox1 (L,N). Arrowheads depict Prox1-positive endothelial cells that appear disorganized in heterozygous (L) but not in wild type control embryos (N).

Figure 8. Gene expression analysis in *Vezf1*$^{-/-}$ embryos.

Semi-quantitative RT-PCR (25, 30, and 35 cycles) using transcript-specific primers was performed on total RNA isolated from E10.5 *Vezf1*$^{-/-}$ and wild type embryos. Primer pairs specific for *Vezf1* and *β-actin* transcripts were included in the analysis as controls, and to normalize for RNA amounts, respectively. *Vezf1* transcripts are absent in *Vezf1*$^{-/-}$ embryos..

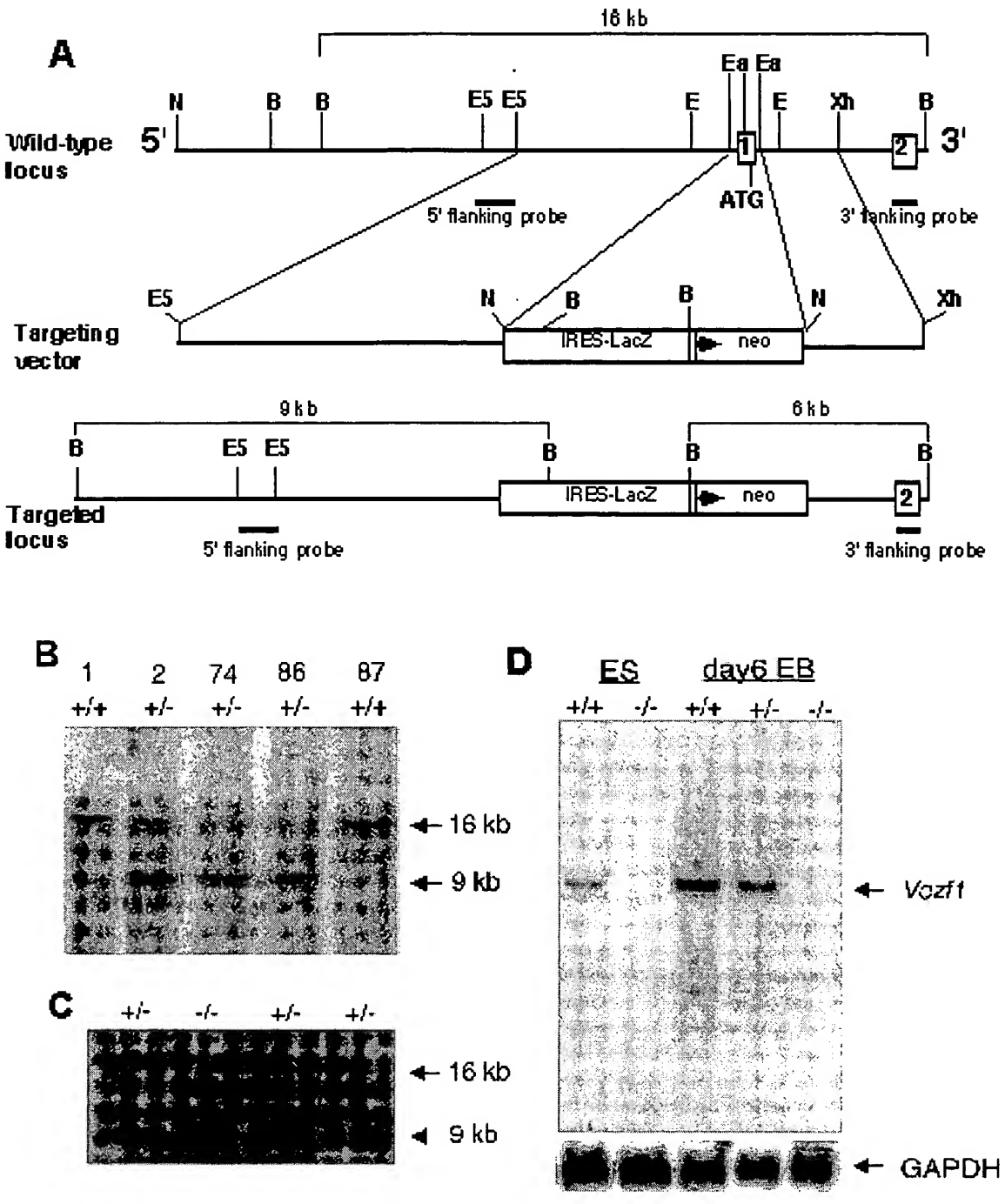
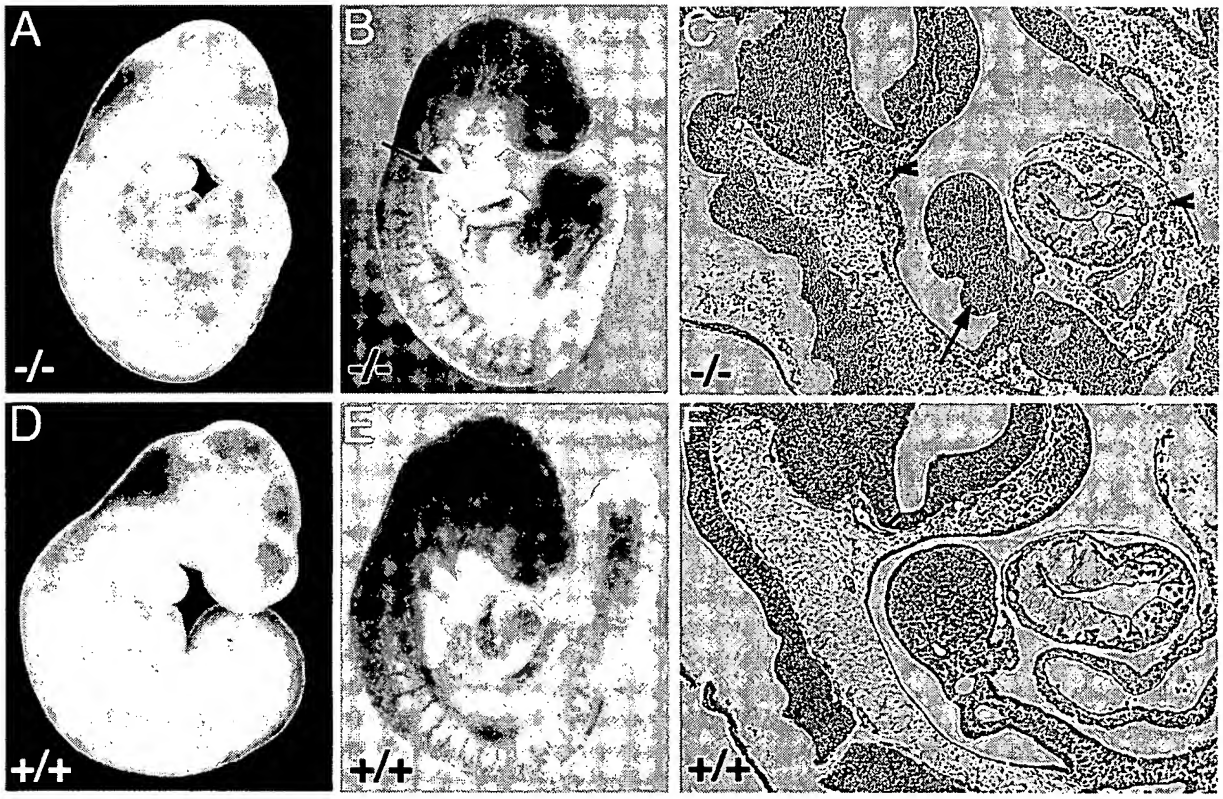


Fig. 1 Kuhnert et al.



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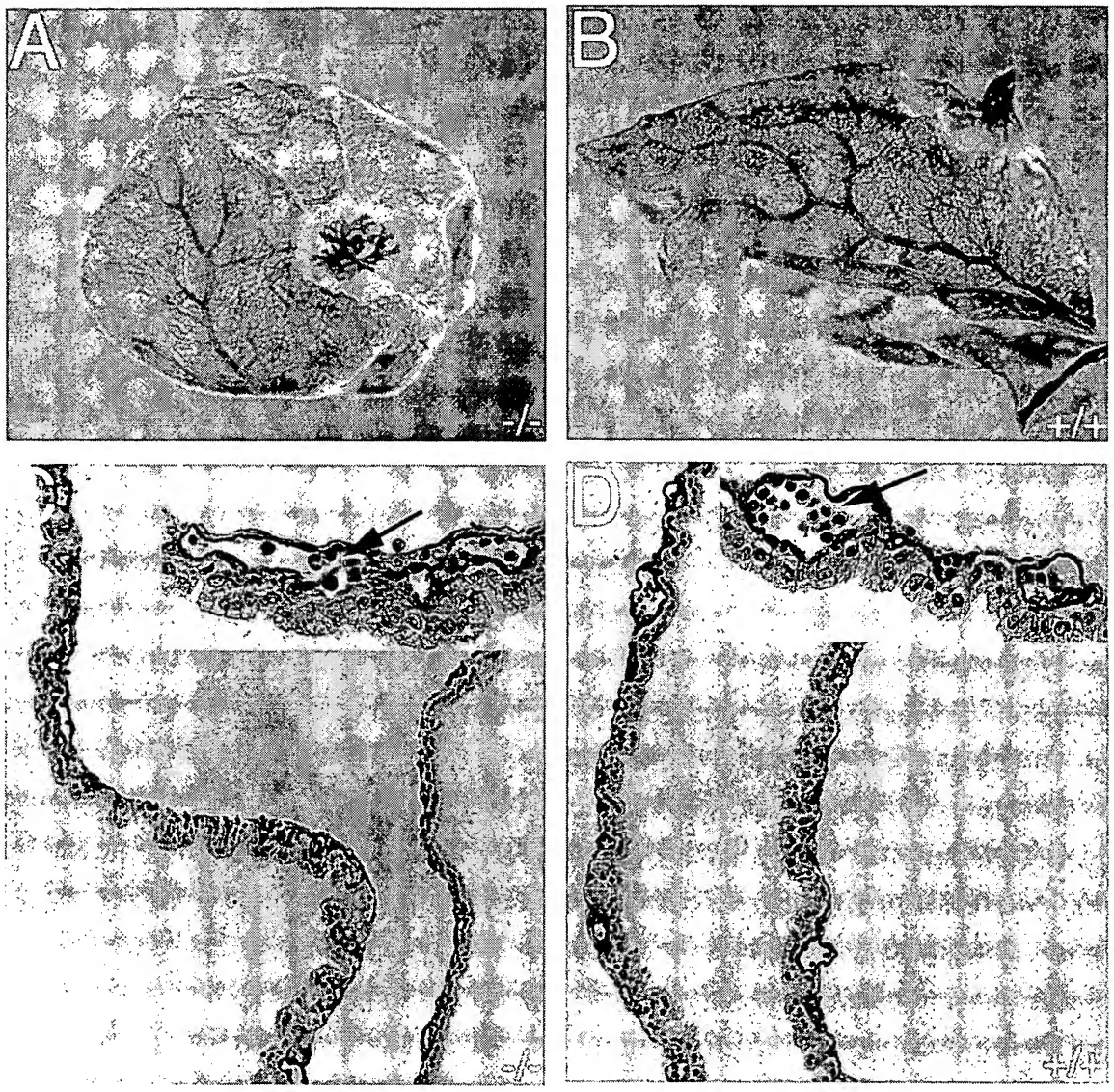

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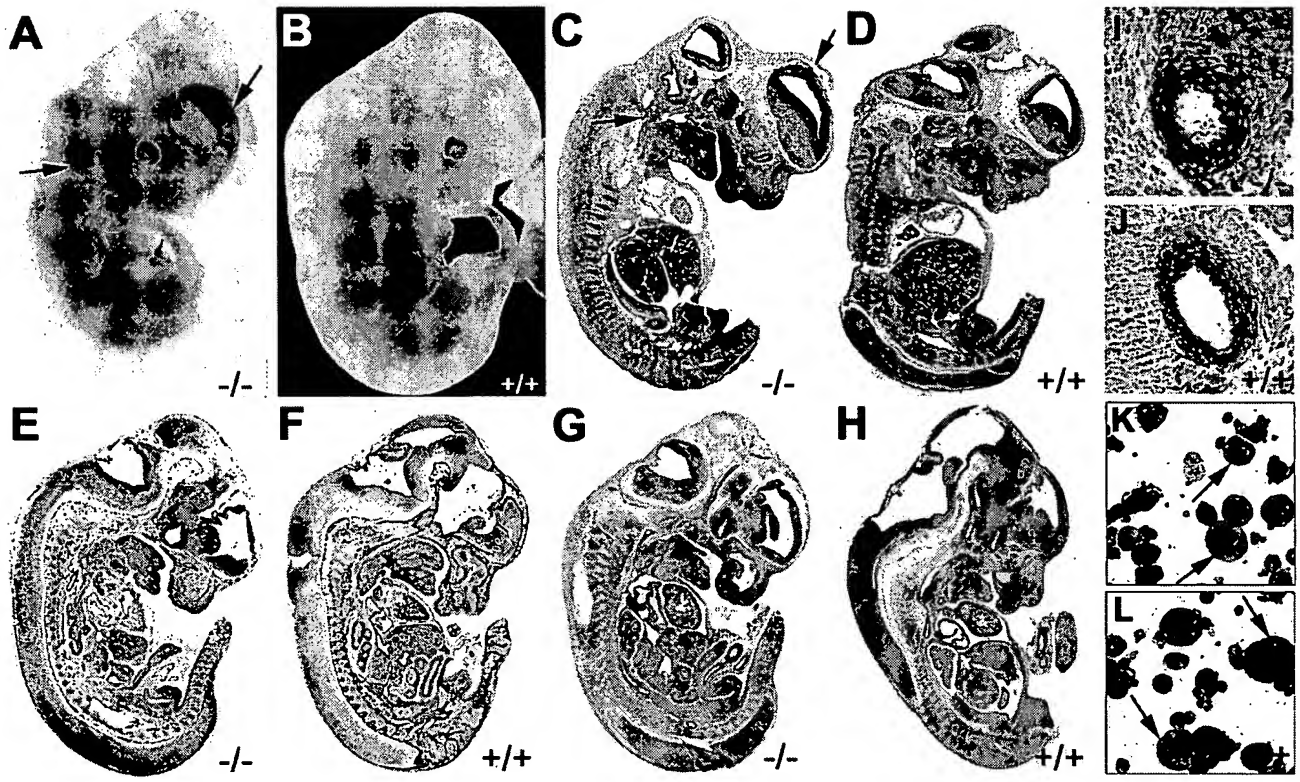

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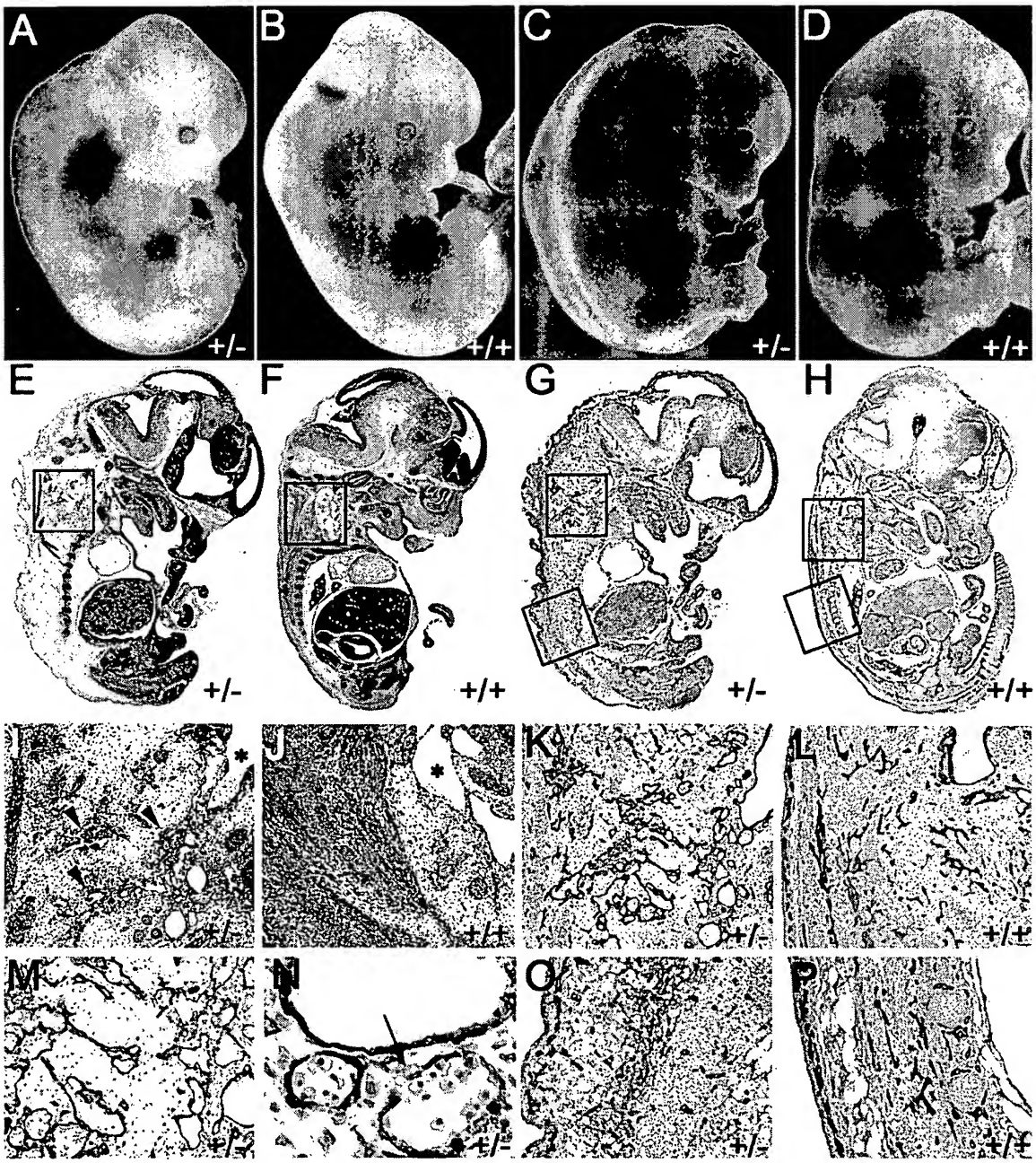
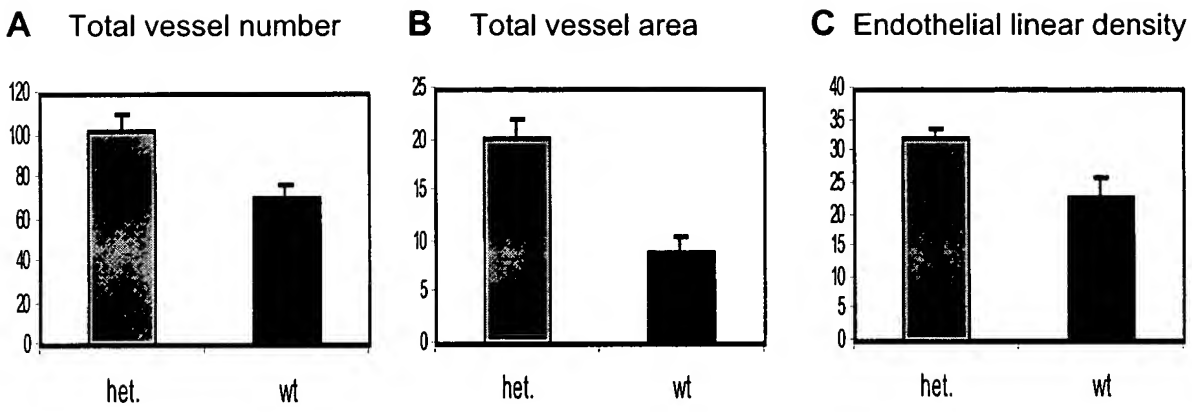


Fig. 5 Kuhnert et al.



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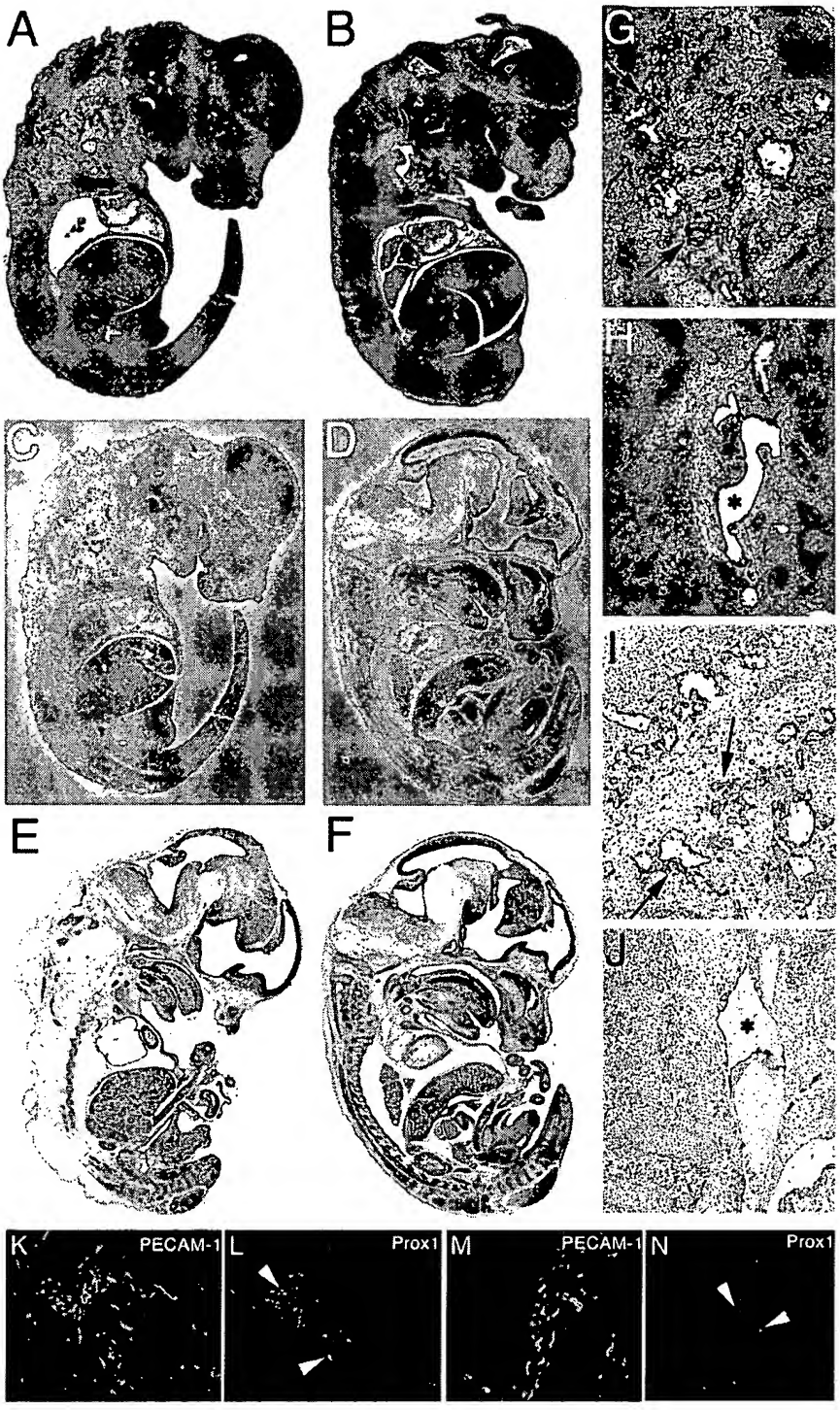

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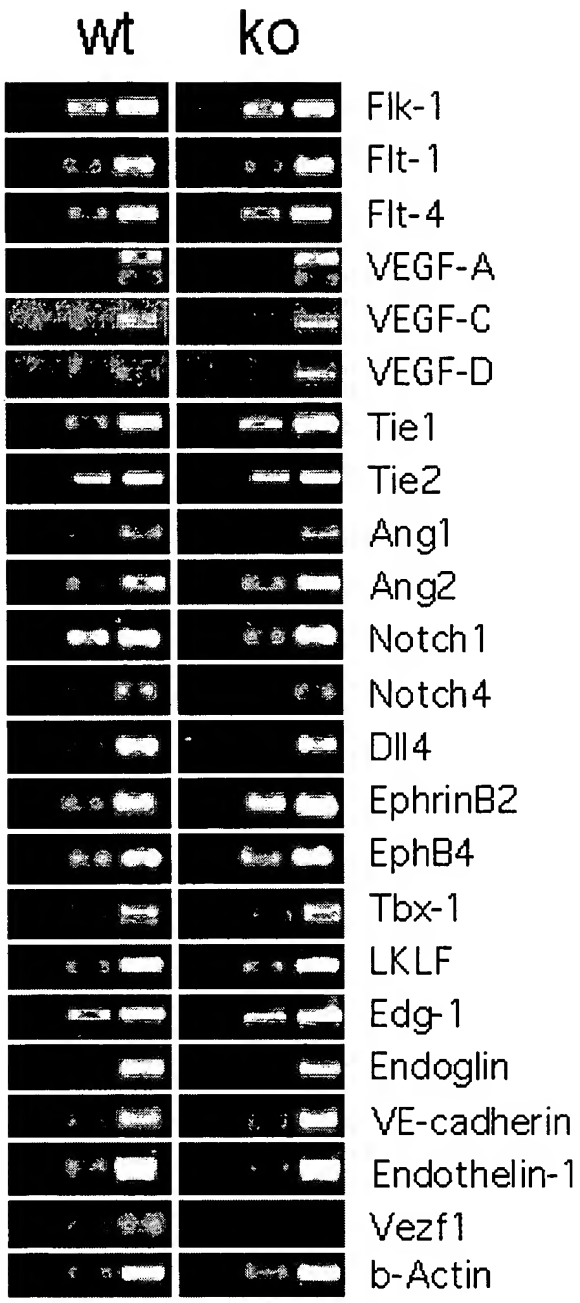

Fig. 8 Kuhnert et al.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Stuhlmann et al.

Serial No.: 09/083,290 Examiner : Wilson, M

Filed : May 22,1998 Group Art Unit: 1633

For :VASCULAR ENDOTHELIAL ZINC FINGER 1 GENE AND PROTEIN AND USES THEREOF

DECLARATION OF DR.HEIDI STUHLMANN

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I, Dr. HEIDI M. STUHLMANN, DECLARE THE FOLLOWING:

1. I am a co-inventor of the above-identified patent application.

2. I am a co-author of the attached manuscript, which describes experiments which (i) investigated the expression of *Vezf1* in the adult vasculature and compared it with that of another endothelial cell marker gene, *flk-1*, and (ii) compared the regulation of these genes in model systems for arterial injury and tumor angiogenesis. Using RNA *in situ* hybridization, *Vezf1* expression in the vasculature was found to be restricted to the endothelium of capillaries and mature vessels. Upon arterial injury, expression of *Vezf1*, but not *flk-1*, was up-regulated in the regenerating endothelium only. During tumor angiogenesis, up-regulation of *Vezf1* was detected throughout the capillary network within tumors induced by human primary carcinoma cell lines grown in nude mice, as well as in metastatic human tumors.

3. Based on the results of experiments described in the attached manuscript, I conclude that *Vezf1* is a marker specific for adult endothelial cells which is expressed normally in

the endothelial cell lining of capillary networks and mature vessels throughout adult tissues. Its expression is upregulated in proliferating endothelial cells during pathological growth, i.e. arterial injury, tumor angiogenesis and atherosclerotic plaque development. Restriction to vascular endothelial cells and upregulation of expression in these cells during normal and pathological proliferation demonstrate that *Vezf1* is a potent marker for angiogenesis. Therefore, *Vezf1* would be useful for identifying endothelial cells and for detecting arterial injury and angiogenesis in histopathologic evaluations. Further, in view of its more uniform expression in endothelial cells from a diversity of tissues and its increased expression in regenerating endothelium , *Vezf1* may be a better marker for endothelial cells than the known endothelial cell marker, *flk-1*.

4. I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that I make these statements with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified patent application or any patent issuing thereon.

Date: November 18, 2001

Signed: Heidi Stuhlmann

Dr.Heidi Stuhlmann

ATCC

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Brookdale Center for Development and Molecular Biology
Mount Sinai School of Medicine
Attn: Heidi Stuhlmann, Ph.D.
Box 1126
New York, NY 10029

Deposited on Behalf of: Mount Sinai School of Medicine

Identification Reference by Depositor:	**ATCC Designation**
Plasmid DNA mVezf1.1	209873
Plasmid DNA mVezf1.2	209874
Plasmid DNA mVezf1.N	209875

The deposits were accompanied by: ___ a scientific description _ a proposed taxonomic description indicated above. The deposits were received May 19, 1998 by this International Depository Authority and have been accepted.

AT YOUR REQUEST: X We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested June 10, 1998. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having authority to represent ATCC:

Barbara M. Hailey

Barbara M. Hailey, Administrator, Patent Depository

Date: June 24, 1998

cc: Dr. Lisa Kole, Esq. (Ref. Docket 970901)